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## ERRATA

- Vol. 1, page 269, line 3 and line 8 from bottom, "427" should read "472"
- Vol. 8, page 95, line 10 from bottom, "7-1/2 months" should read "7-1/2 weeks"
- Vol. 12, page 326, line 3, "(3) 0.6 mg./g." should read "(3) 0.06 mg./g."
- Vol. 13, page 91, line 16, "0.01" should read "0.02"
- Vol. 13, page vi, line 1 under ADDENDA, "Figure 7" should read "Figure 2"
- Vol. 14, page 96, Table I, column 2, line 13, "190" should read "180"
- Vol. 14, page 100, Table V, column 3, line 7, "0.003" should read "Inactive"
- Vol. 14, page 161, footnote 2, "Carbide and Carbon Company" should read "Carbide and Carbon Chemicals Corporation"
- Vol. 14, page 262, line 32, "333 cc." should read "33 cc."
- Vol. 14, page 339, Figure 2, for "K" substitute "L" and vice versa
- Vol. 14, page 340, line 4 should read "than the coarser." Delete "so that one would expect the value for  $M_v'$  to be the higher of the two. This was the case in four out of the five instances where both constants were determined."

## ADDENDA

- Vol. 13, page 512, below line 10 add "*Note Added in Page Proof*"

## SYNERGISTIC EFFECTS OF THREE CHEMICALS IN THE TREATMENT OF DORMANT POTATO TUBERS TO HASTEN GERMINATION

F. E. DENNY

Previous experiments had shown the effectiveness of the vapor of ethylene chlorohydrin ( $\text{CH}_2\text{ClCH}_2\text{OH}$ ) in hastening the germination of the dormant tubers of potato (*Solanum tuberosum* L.), and had indicated that when the intact tubers were to be treated, as distinguished from the treatment of cut tubers, the proper amount of chemical to use (2, p. 8) was 1 cc. of the 40 per cent solution of ethylene chlorohydrin per pound of potato tubers treated, or about 2.2 cc. of the 40 per cent, or about 0.8 cc. of the anhydrous chemical per kilogram of tubers.

Thus, the volatilization of about 60 cc. of the 40 per cent solution, or about 22 cc. of the anhydrous chemical is required per bushel of tubers, and since the boiling points are rather high,  $128.7^\circ\text{C}$ . for the anhydrous and  $97.8^\circ\text{C}$ . for the 40 per cent solution, the evaporation is not rapid. Although these amounts of chemical are volatilized completely during the four-day period of treatment, it was considered desirable to determine whether a portion of the requirement for ethylene chlorohydrin could be substituted for by means of some other chemical which was more volatile, which would produce an effect at lower concentrations, and which was miscible with the anhydrous ethylene chlorohydrin.

Among the chemicals previously tested and which were considered as possible adjuvants for chlorohydrin were ethylene dichloride ( $\text{CH}_2\text{ClCH}_2\text{Cl}$ ) and carbon tetrachloride ( $\text{CCl}_4$ ), each of which was found to hasten germination of dormant tubers. For use as a volatile insecticide, Cotton and Roark (1) had recommended a mixture of these two chemicals in the proportion of three parts by volume of the dichloride to one part of the tetrachloride, the advantage being that the vapor of the combination was non-inflammable and non-explosive. This mixture of the two chemicals was tested with dormant potato tubers and was found to have a favorable effect.

The combination of chemicals finally adopted for hastening the germination of recently-harvested potato tubers *in this experiment* was one consisting of seven parts by volume of anhydrous ethylene chlorohydrin, three parts of ethylene dichloride and one part of carbon tetrachloride. As a convenient laboratory name for this mixture, the word "rindite," obtained by using one syllable from the names of each of the three components, was used.

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The object of the present report is to show the results obtained by treating dormant potato tubers with various concentrations of "rindite" and to compare them with the results obtained by treating tubers with various concentrations of each one of the three components of "rindite."

The sprouting responses of the treated tubers showed that the object originally sought was attained, i.e., the addition of small amounts of the dichloride and tetrachloride made it possible to reduce the amount of chlorohydrin needed to produce a favorable result.

The question then arose whether the response obtained with the combined chemical was greater than the sum of the responses obtained by the three separate components, i.e., whether the effects of the chemicals were synergistic. The word "synergism" seems to be defined differently by different authors, but the definition that will be used here is the one given in Webster's Unabridged dictionary (5) and is as follows: "cooperative action of discrete agencies such that the total effect is greater than the sum of the two effects taken independently."

Two different methods of computation were used in arriving at a measure of the sum of the effects of the three chemicals, so as to make comparisons with the effect of the combined chemical. By both methods there was obtained evidence that the action of the combined chemical, "rindite," was greater than the sum of the effects of the three components and, furthermore, there was some evidence that this was obtained by a sort of "cooperation" among the constituents, thus fulfilling the requirements of the definition of synergism given above.

The combined chemical gave a result at least as good as could be obtained with the most effective of the three (ethylene chlorohydrin) acting alone, and at a cost of about one-third for the chemical needed.

#### MATERIALS AND METHODS

The tubers were grown in the Institute gardens and were harvested August 7 to 9, 1944. They were sorted into three sizes, the tubers averaging 5, 3, and 2 ounces (approx. 142, 85, and 57 g. respectively), and in taking samples for the treatments at intervals later the proportion of sizes shown by this sorting at harvest time was adhered to. In one test (lot G), a still smaller size was used, i.e. 1.5 ounces (42.5 g.).

The treatments were made in one-gallon or two-gallon glazed earthenware jars fitted with covers either of the same material or of glass, a tight seal being obtained with modeling clay. The tubers in the containers were covered with a paper towel and on this was spread loosely pieces of cheesecloth in which was incorporated the proper amount of chemical as determined by the weight of tubers in the jar, using enough cloth to prevent excessive dripping. The containers were then sealed and stored in a room held at 22.5° C. (72.5° F.), with a deviation of not more than 1° C. at any

time. At the completion of the treatment, the tubers were stored in paper bags for two days at room temperature, varying from about 20° to 30° C. (or 68° to 86° F.), but usually about 25° to 28° C. (77° to 82.4° F.) before being planted. The treated tubers were then cut into seed-pieces, at least one piece from each tuber, more than one piece per tuber being needed in many cases in order to obtain the required number, which was 50 pieces, for each lot. The controls, or untreated lots, were merely held in paper bags adjacent to the treated lots in the storage space, and samples were taken from these lots in the manner just described.

The seed-pieces were planted in soil in wooden flats, and were examined at least twice each week, in order to maintain the proper amount of mois-

TABLE I  
AMOUNT OF EACH CONSTITUENT TAKEN WHEN VARIOUS AMOUNTS OF THE  
COMBINED CHEMICAL WERE TAKEN

Combined chemical, "rindite," cc.	Constituents of "rindite"		
	Ethylene chlorohydrin, anhydrous, cc.	Ethylene dichloride, cc.	Carbon tetrachloride, cc.
0.8	0.5091	0.2182	0.0727
0.5	0.3182	0.1364	0.0454
0.3	0.1909	0.0818	0.0273
0.2	0.1273	0.0545	0.0182

ture and to obtain a count of sprouts. As soon as a sprout of any lot emerged, it was recorded and discarded. This gave a record of the germination time for each sprout and permitted the determination of the average time for emergence of sprouts in each lot. Treatments showing rotting of seed-pieces to such an extent that 90 per cent emergence was not obtained were recorded as having been unsuccessful.

The amounts of each of the three components which were taken when various amounts of "rindite" were used in the treatments are shown in Table I, tests having shown that no volume change occurred when the chemicals were mixed.

## RESULTS

The average number of days for the emergence of sprouts in each test is shown in Table II. Both ethylene chlorohydrin and "rindite" were effective in shortening the germination time and could be applied at a considerable range of concentration without producing injury.

Ethylene chlorohydrin was tested in each experiment with the next highest concentration in the series, i.e., at 1.3 cc. per kg. With lots C and E, this amount was injurious, and with the other lots the ratios, average

TABLE II

EFFECT OF THE COMBINED CHEMICAL, "RINDITE," AND EACH OF ITS CONSTITUENT CHEMICALS UPON THE GERMINATION OF DORMANT POTATO TUBERS

Lot No. and variety	Av. days for germ. of controls	Days after harvest	Concn. of chemical, cc./kg. of tubers	Average number of days required for emergence of sprouts			
				Combined chemical, "rindite"	Ethylene chlorohydrin	Ethylene dichloride	Carbon tetrachloride
A Irish Cobbler	50.7	3	0.8	15.3	12.1	*	—
			0.5	11.8	13.1	*	*
			0.3	10.8	14.2	34.8	31.8
			0.2	14.2	20.2	24.8	—
			0.1	—	—	—	37.3
B Katahdin	84.0	6	0.8	21.4	13.4	32.5	—
			0.5	15.7	17.0	58.3	*
			0.3	18.0	23.4	61.0	57.5
			0.2	—	23.0	68.1	71.5
			0.1	—	—	—	84.5
C Irish Cobbler	52.4	8	0.8	16.6	14.2	*	—
			0.5	13.5	18.6	31.3	*
			0.3	14.0	30.8	36.8	38.1
			0.2	19.0	29.6	44.5	46.8
			0.1	—	—	—	—
D Early Ohio	70.6	10	0.8	24.1	19.9	*	—
			0.5	17.9	27.6	43.3	*
			0.3	16.9	32.6	42.2	48.4
			0.2	19.9	47.9	40.9	50.2
			0.1	—	—	—	60.6
E Irish Cobbler	31.3	15	0.8	15.5	12.6	*	—
			0.5	12.4	19.3	*	*
			0.3	12.3	20.3	28.4	24.5
			0.2	20.6	20.7	28.4	23.6
			0.1	—	—	—	31.7
F Katahdin	50.0	16	0.8	17.1	13.0	*	—
			0.5	13.5	13.5	29.0	*
			0.3	12.6	15.6	36.9	*
			0.2	12.4	16.3	36.9	31.2
			0.1	—	—	—	39.2
G Irish Cobbler, small size	46.3	21	0.8	12.4	13.4	*	22.8
			0.5	12.1	15.2	28.9	21.2
			0.3	11.8	15.7	28.8	26.7
			0.2	—	21.1	—	34.4
			0.1	—	—	—	—
H Irish Cobbler	30.8	21	0.8	13.6	12.8	*	—
			0.5	12.8	13.4	24.2	18.2
			0.3	12.8	14.1	21.8	23.5
			0.2	12.8	18.9	21.1	20.9
			0.1	—	—	—	27.2
K Katahdin	26.1	26	0.8	13.4	13.4	21.8	—
			0.5	12.5	14.4	24.2	18.8
			0.3	12.3	16.0	25.4	19.0
			0.2	13.8	15.7	24.1	19.2
			0.1	—	—	—	20.9

\* Injury resulted at this concentration.

days for germination for lots treated with 1.3 cc. ÷ days for 0.8 cc., were 1.12, 1.10, 1.06, 1.00, 0.92, 1.01, and 1.04. The average of these ratios is 1.04. It is likely that the most favorable concentration for ethylene chlorohydrin is at 1.0 cc. of the anhydrous chemical per kg. of tubers.

The ethylene dichloride and carbon tetrachloride were much less effective when applied separately, requiring even at the most favorable concentration usually at least twice as long a period for germination as was obtained with ethylene chlorohydrin or "rindite," and also were less consistent as to the amount that could be applied without producing injury. Although the ranges of effectiveness for the dichloride and the tetrachloride were narrow, small amounts of these chemicals had some effect, and when they were incorporated into the chlorohydrin to give "rindite," it is seen that low concentrations of the combined chemical were quite effective in hastening germination. Thus, in most cases 0.3 cc. of "rindite" per kg. of tubers gave as favorable an effect as was obtained with 0.8 cc. of ethylene chlorohydrin, and similarly 0.2 cc. of "rindite" was approximately equal to 0.5 cc. of ethylene chlorohydrin.

Now, since as shown in Table I, 0.3 cc. of "rindite" contained less than 0.2 cc. of chlorohydrin, less than 0.1 cc. of dichloride, and less than 0.03 cc. of tetrachloride, and likewise 0.2 cc. of "rindite" was equivalent to less than 0.13 cc., 0.055 cc., and 0.02 cc. respectively of the chlorohydrin, dichloride, and tetrachloride, the question arose whether the effectiveness of the combined chemical was greater than could be expected from the combined effects of these components taken separately, i.e., whether a synergistic effect was obtained by combining the chemicals.

#### METHODS OF ESTIMATING THE ADDITIVE EFFECTS OF SEPARATE COMPONENTS

Whether the effect of a chemical prepared by combining three chemicals is greater than the combined effects of the three chemicals each acting separately will depend, of course, on how the addition of the separate effects can be made. The attempt here made is based on the additive effects, either as read from curves showing the relation between the amount of each chemical and the effect produced by that amount, or as computed from algebraic equations fitted to the observed points.

This may be done from two different points of view: 1. By determining the effect of each amount of chemical separately, as though the given amount of each component was acting from the point of zero chemical on curve. This would be the maximum effect that such amounts of chemical could exert, since all of the curves were changing relatively most rapidly when small amounts of chemical were added. The sum of gains due to the three components taken separately was compared with that obtained with the corresponding amount of the combined chemical. This method of add-

ing the separate effects is called the "independent summation" method. 2. By computing the change progressively on each curve as larger and larger amounts of chemical were added. Thus, the amount of tetrachloride added was observed in a given case to produce a certain gain in the time of sprouting; from this point on the tetrachloride curve a transfer was made to the equivalent point on the dichloride curve, and the quantity of dichloride corresponding to this point was noted by reference to the base line; to this amount was added the amount of dichloride present in the portion of the combined chemical added in that particular test as shown in Table I; from the sum of these two the additional gain due to the added amount of dichloride could be read from the curve. Again transferring from the dichloride curve to the chlorohydrin curve, the gain due to the additional amount of chlorohydrin could be computed in the same way. The sum of these three contributions made successively on the curves from point to point indicates the additive effect of the three separate components, and this is compared with the gain due to the given amount of the combined chemical. This method of computing is called the "consecutive summation" method. The order in which the chemicals are taken in obtaining the consecutive summations was of some importance, and in this report the order chosen for reporting is that order in which the largest value was obtained for the summation of the three individual components. Ethylene chlorohydrin could not be used as a starting component because its contribution, even with the smallest amount, brought its curve down to a point below either one or both of the other chemicals, making impossible a transfer from this point on its curve to equivalent points on the other curves.

The description of the methods thus far refers to the procedure when graphs only are available. The same procedure is applicable also when equations for the curves are used. In such a case the various points on the curve are located quite accurately by substituting the proper values in each of the equations.

#### COMPUTATIONS MADE BY INTERPOLATING ON GRAPHS

The data for each of the four chemicals in each of the nine lots in Table II were plotted on cross-section paper, using large sheets, 22" X 17", graduated to tenths of an inch, adjusting the values of the base line showing cc. of chemical, and those of the ordinates showing days required for germination, so as to spread the curves over the area of the paper. Curves were drawn to pass through these points as nearly as could be estimated using large-size French curves.

Interpolations on these curves were made to determine the gain in germination time due to the combined chemical, "rindite," and due to each of the separate constituents by both the "independent summation" and

"consecutive summation" methods. The sum of the gains due to the individual chemicals was compared to the gain due to the combined chemical and the results are shown in Table III.

To illustrate how the summations were made we may take the results due to 0.2 cc. of "rindite" in the test with lot A in Table II. From Table I it is seen that in adding 0.2 cc. of "rindite" per kg. of tubers there are added

TABLE III

A COMPARISON OF DAYS GAINED IN RATE OF GERMINATION OF DORMANT POTATO TUBERS BY TREATMENT WITH THE COMBINED CHEMICAL, "RINDITE," AND WITH THE SEPARATE COMPONENTS, ETHYLENE CHLOROHYDRIN, ETHYLENE DICHLORIDE, AND CARBON TETRACHLORIDE

Lot No.	When 0.2 cc. of combined chemical ("rindite") was used					When 0.3 cc. of combined chemical ("rindite") was used				
	Days gained by treatment					Days gained by treatment				
	Combined chemical, "rindite"	Independent summations		Consecutive summations		Combined chemical, "rindite"	Independent summations		Consecutive summations	
		Separate chemicals	Diff.	Separate chemicals	Diff.		Separate chemicals	Diff.	Separate chemicals	Diff.
A	30.5	32.6	+ 3.9	28.2	+ 8.3	39.9	43.6	+ 3.7	35.2	+ 4.7
B	51.0	49.5	+ 1.5	47.0	+ 4.0	60.0	57.5	+ 8.5	55.0	+11.0
C	33.4	16.2	+17.2	16.5	+16.9	38.4	21.6	+16.8	21.9	+16.5
D	50.7	29.8	+20.9	28.1	+22.6	53.7	43.0	+10.7	37.6	+19.1
E	10.7	10.1	+ 0.6	9.8	+ 0.9	19.0	12.6	+ 6.4	11.3	+ 7.7
F	37.6	34.6	+ 3.0	30.5	+ 7.1	37.4	41.4	+ 4.0	34.0	+ 3.4
G	30.8	26.3	+ 4.5	23.0	+ 7.8	34.5	34.6	+ 0.1	28.8	+ 5.7
H	18.0	12.1	+ 5.9	12.8	+ 5.2	18.0	17.6	+ 0.4	16.5	+ 1.5
K	12.3	8.0	+ 4.3	7.7	+ 4.6	13.8	11.2	+ 2.6	10.3	+ 3.5
Average			+ 6.87		+ 8.60		+ 4.17		+ 8.12	
S.E.			2.370		2.285		2.326		2.056	
Sign. at .05 level			2.809		3.763		1.793		3.050	
" " .01 " "			+		+		—		+	
			—		+		—		+	

Note: Values in column 4 are column 2 minus column 3; in column 6, column 2 minus column 5; in column 9, column 7 minus column 8; and in column 11, column 7 minus column 10.

the following amounts of the separate components (to the nearest decimals suitable for reading from the graph): 0.13 cc. of chlorohydrin, 0.055 cc. of dichloride, and 0.02 cc. of carbon tetrachloride. The ordinate corresponding to 0.13 cc. of chlorohydrin was 28.0 days and the gain due to that amount of chlorohydrin was  $50.7 - 28.0 = 22.7$  days. The ordinate corresponding to 0.055 cc. of dichloride was 44.0 days and the gain due to that amount of dichloride was  $50.7 - 44.0 = 6.7$  days. The ordinate for 0.02 cc. of carbon tetrachloride was 47.5 days and the gain was  $50.7 - 47.5 = 3.2$  days. The sum of the gains due to the chemicals taken separately was 22.7



+6.7+3.2=32.6 days. The gain due to the combined chemical, "rindite," was  $50.7 - 14.2 = 36.5$ . This result is by the "independent summation" method and the values are shown in Table III, line 1. The computation by the "consecutive summation" method is as follows. The gain due to 0.02 cc. of carbon tetrachloride was  $50.7 - 47.5 = 3.2$  days. The base line value corresponding to 47.5 days on the dichloride curve was 0.022 cc., and since 0.055 cc. of dichloride was added in the 0.2 cc. of "rindite" we need to find the ordinate on the dichloride curve corresponding to 0.077 cc., which was found to be 40.5 days, and the gain due to the dichloride added was  $47.5 - 40.5 = 7.0$  days. The base line value corresponding to 40.5 days on the chlorohydrin curve was 0.048 cc., and since 0.13 cc. of chlorohydrin was added in the 0.2 cc. of "rindite," we need to find the ordinate corresponding to 0.178 on the chlorohydrin curve, which was 22.5 days, and the gain due to the added chlorohydrin is  $40.5 - 22.5 = 18.0$  days. The sum of the gains contributed by tetrachloride, dichloride, and chlorohydrin was  $3.2 + 7.0 + 18.0 = 28.2$  days. The gain due to 0.2 cc. of "rindite" was  $50.7 - 14.2 = 36.5$  days. These entries are found in Table III, line 1, under the entry consecutive summations.

In like manner were computed the other values in Table III for both 0.2 cc. and 0.3 cc. of "rindite." The difference between the days gained by the combined chemical, "rindite," and the sums of the days gained by the separate components are shown in columns 4, 6, 9, and 11 in Table III. The S.E. values in Table III were computed by Student's method (4, p. 22), and the significance of the  $t$  values obtained by dividing the average difference by the S.E. values was obtained by reference to the  $t$  table (4, p. 248). When the comparison is made on the basis of the effect of 0.2 cc. of "rindite" per kg. of tubers the evidence is good that the effect of the combined chemical was greater than the sum of the component effects, whether the summation was made on the independent or the consecutive basis of computing. By the consecutive summation method the odds were more than 99 to 1. When the amount of "rindite" employed was 0.3 cc. per kg. of tubers the consecutive summation method also showed high odds in favor of the combined chemical, but by the independent summation method the difference does not reach the 0.05 level, the odds being in this case about 8 to 1.

#### COMPUTATIONS MADE ON EQUATIONS FITTED TO THE CURVES

##### *Including All Lots, A to K Inclusive*

In order to avoid the uncertainties involved in graphical interpolation from more or less free-hand curves the attempt was made to find equations which would satisfactorily represent the relation between the amount of the chemical used and the sprouting response obtained. To have found such

equations for each of the 36 curves needed to represent the data in Table II would have required an excessive and probably unjustified amount of work. Therefore, a method of combining the values for all of the different lots in Table II into four sets of values, one set for each of the four chemicals, was used. To do this each value in the body of Table II was expressed as the percentage of the corresponding control value for that lot as shown by the value in column 2 of Table II. These percentages were then averaged (omitting the relatively few values obtained for 0.8 cc. of dichloride, and 0.8 and 0.5 cc. of tetrachloride). From seven to nine values were thus

TABLE IV  
DATA IN THE BODY OF TABLE II EXPRESSED AS A PER CENT OF THE CORRESPONDING CONTROL LOT, AND AVERAGED FOR ALL LOTS

Concn. of chemical, cc./kg.	"Rindite"		Ethylene chlorohydrin		Ethylene dichloride		Carbon tetrachloride	
	Obs.	Calcd.	Obs.	Calcd.	Obs.	Calcd.	Obs.	Calcd.
0.8	37.5	37.3	31.5	31.8	—	—	—	—
0.5	31.0	30.9	37.9	37.8	69.0	69.5	—	—
0.3	30.2	30.2	44.1	44.3	73.7	72.0	69.6	69.6
0.2	37.9	38.3	51.0	51.8	75.0	75.5	74.7	74.7
0.1	—	—	—	—	—	—	86.6	86.6

Note: Obs. means values obtained by averaging the observed percentages, and calcd. means values obtained by substitution in the equations shown in the text.

available for obtaining each average for each concentration of each chemical. The observed averages are shown in Table IV, columns 2, 4, 6, and 8. Each average value was based on the germination counts of 315 to 450 potato-tuber cuttings.

These are the basic data from which the graph in Figure 1 was prepared. The empirical equation used for all of the chemicals except carbon tetrachloride was of the form:  $1000x \div (y - 100) = a + bx + cx^2 + dx^3$ , where  $x$  is the concentration of the chemical expressed as cc. per kg. of tubers,  $y$  is the per cent of the control value (which was 100), and  $a$ ,  $b$ ,  $c$ , and  $d$  were constants which had to be determined from the data. For carbon tetrachloride a more suitable equation was of the form:  $y = a + bx + cx^2 + dx^3$ . The following equations were found to give a satisfactory fit:

$$\text{For "rindite": } 1000x \div (y - 100) = -2.219 - 0.838x - 23.3x^2 + 9.87x^3$$

$$\text{For chlorohydrin: } 1000x \div (y - 100) = -2.105 - 8.23x - 11.54x^2 + 8.5x^3$$

$$\text{For dichloride: } 1000x \div (y - 100) = -4.424 - 15.14x - 18.96x^2 + 2.27x^3$$

$$\text{For tetrachloride: } y = 100 - 123.8x - 190x^2 + 883x^3$$

The curve in Figure 1 was drawn by computing the points for 0.1, 0.2, 0.3, etc. for each chemical, and the experimentally observed average values were plotted at the appropriate points on this curve.

When 0.2 cc. of "rindite" was used per kg. of tubers the  $x$  value for computing from the above equations was 0.2, and the corresponding  $x$  values for chlorohydrin, dichloride and tetrachloride are found in line 4 in the body of Table I. Substitution in the equations gave the percentage values corresponding to each of these amounts, and the gain due to each chemical was found by subtraction from the control value of 100. Applying the independent summation method as previously described for the

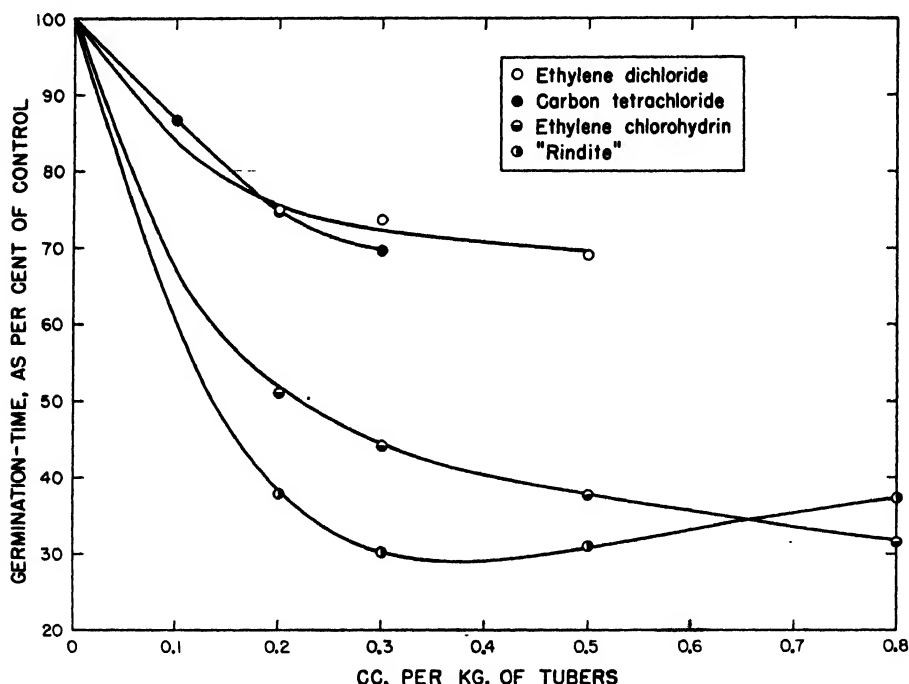


FIGURE 1. Germination-time values for each of the entries in Table II expressed as a percentage of the corresponding control value for each lot of tubers and then averaged for each concentration of each chemical. Each plotted point was the average obtained from the germination counts of 315 to 450 potato-tuber cuttings. Plotted points obtained from observed germinations, curves from empirical equations fitted to these points.

free-hand curves, the sum of the gains due to the three chemicals considered separately was 50.9, while that due to the combined chemical was 61.9, these values being in units of the plotting scale in Figure 1.

With computations by the consecutive summation method previously described, the transfer from one curve to another at the same level of ordinates is not as easy with the equations as with the graphs, because one needs to know the value of  $x$  for a given value of  $y$ . However, by finding the approximate  $x$  value from the curve and substituting one or two values on

either side of this point and then interpolating, a suitable  $x$  value was found and the fact that this was so was verified by substituting in the equation. When the values for "rindite" at 0.2 cc. per kg. were computed by the consecutive summation method, the value corresponding to the sum of the gains due to the three components was 43.7, and that due to "rindite" was 61.9.

When 0.3 cc. of "rindite" was used per kg. of tubers the amounts of the three component chemicals used are shown in line 3 in the body of Table I. Computations by the independent summation method showed that the sum of the gains due to the three components was 63.9, and that due to "rindite" was 69.8, and by the consecutive summation method the corresponding values are for the sum of the three components, 51.9, and for "rindite," 69.8.

*Including Only Lots C, D, G, H, and K*

There were some missing entries in Table II, and so the averages in Table IV, and the curves in Figure 1, although based on nearly the same

TABLE V

DATA FROM LOTS C, D, G, H, AND K IN TABLE II, EXPRESSED AS A PER CENT OF THE CORRESPONDING CONTROL LOT, AND AVERAGED FOR ALL LOTS

Concn. of chemical, cc./kg.	"Rindite"		Ethylene chlorohydrin		Ethylene dichloride		Carbon tetrachloride	
	Obs.	Calcd.	Obs.	Calcd.	Obs.	Calcd.	Obs.	Calcd.
0.8	39.6	40.0	35.4	35.5	—	—	—	—
0.5	33.6	32.0	41.2	40.4	71.2	71.2	—	—
0.3	33.2	32.5	49.0	49.6	72.0	72.0	69.6	69.3
0.2	30.8	39.0	58.4	58.3	—	—	75.2	75.2

number of total entries in each case are not exactly balanced as to the number of entries from the different lots of tubers. In order to put the entries in balance by obtaining comparable representation from each lot of tubers, a new table was prepared in which the data of only lots C, D, G, H, and K were used. By using the values for 0.8, 0.5, 0.3, and 0.2 cc. for "rindite" and ethylene chlorohydrin, 0.5 and 0.3 cc. for ethylene dichloride, and 0.3 and 0.2 cc. for carbon tetrachloride, a complete table without any missing spaces was obtained, 5 entries being available for each of the 12 columns available. A new set of averages for the different concentrations of the four chemicals was obtained and the result is shown in Table V, columns 2, 4, 6, and 8. Curves were plotted, and while they are not shown here, they resembled those in Figure 1, not only in shape but also as to the numerical values of the observed points.

A new set of empirical equations was computed, and they are as follows:

For "rindite":  $1000x \div (y - 100) = -1.44 - 7.641x - 7.374x^2 - 2.067x^3$

For chlorohydrin:  $1000x \div (y - 100) = -2.592 - 10.74x - 1.4x^2 - 0.63x^3$

For dichloride:  $1000x \div (y - 100) = -3.886 - 33.6x - 70x^2 - 113.4x^3$

For tetrachloride:  $y = 100 - 75.34x - 550x^2 + 1533x^3$

The calculated values are shown in Table V, columns 3, 5, 7, and 9.

These also were computed by both the independent summation and consecutive summation methods to determine the sum of the gains by the three components of "rindite" and those due to "rindite" alone. The results were as follows: By the independent summation method, with 0.2 cc. of "rindite" per kg., the gain was 61.0 units and the sum of gains due to the three components was 44.2 units; the corresponding values by the consecutive summation method were: "rindite," 61.0, and the sum of the three, 37.5. With 0.3 cc. of "rindite," the independent summation showed "rindite," 67.5, the sum of the three, 56.3; the consecutive summation method showed "rindite," 67.5, the sum of the three, 46.2

#### POSSIBLE CAUSE OF THE SYNERGISTIC EFFECT

Table II and Figure 1 show not only that the range of concentration for ethylene dichloride and carbon tetrachloride was relatively narrow, but that even when the most favorable concentration was found, neither of these chemicals acting alone was able to produce a satisfactory gain, this being generally less than 50 per cent of the gain shown by favorable amounts of ethylene chlorohydrin or "rindite," and, as the computations show, small amounts of these two chemicals acting alone had little effect. Yet when these small amounts were added to ethylene chlorohydrin they appeared to exert considerable effect in improving the action of the chlorohydrin.

The two chemicals, ethylene dichloride and carbon tetrachloride, were selected at the start of the experiment as possible adjuvants for ethylene chlorohydrin because of the favorable results that had been obtained with them in previous experiments, but these had been carried out mainly not with intact tubers as in the present experiments, but with tubers cut into pieces before treatment. As soon as the disappointing effect in the present tests of the dichloride and tetrachloride acting alone on whole tubers was noted, time was available for three separate tests of these two chemicals with cut tubers in comparison with chlorohydrin and "rindite" on the same stock of cut tubers. In these tests with cut tubers the ratios of the days gained by favorable concentrations of dichloride and tetrachloride to the average days gained by chlorohydrin and "rindite" were for ethylene dichloride: 0.71, 0.85, and 0.64, and for carbon tetrachloride: 0.85, 0.90, and 0.69, indicating that under these conditions the dichloride and tetrachloride were about 75 per cent as effective as chlorohydrin and

"rindite." This indicates that when the tissue was opened up so that the dichloride and tetrachloride could enter, their effect was increased, and suggests that in the treatment of the intact tuber there was insufficient penetration by these two chemicals.

Possibly, then, one of the functions of the chlorohydrin in the mixture called "rindite" is to increase the permeability of the potato, and facilitate the entrance of the dichloride and tetrachloride. If this is the case then it is not the function of the dichloride and tetrachloride to improve the chlorohydrin as mentioned in the second preceding paragraph, but rather it is the function of the chlorohydrin to improve the action of the dichloride and tetrachloride.

This would also be in conformity to the phrase "cooperative action" in the definition of synergism previously quoted, the cooperation being on the basis that the chlorohydrin facilitated the entrance of the dichloride and tetrachloride, and that these then produced an effect in addition to that of the chlorohydrin already present.

That the treatment of potato tubers with ethylene chlorohydrin does increase the permeability of the tissue is indicated by the results of Guthrie (3), who found that the conductivity of the tissue and the rate of leaching of electrolytes was increased by the treatment with ethylene chlorohydrin.

#### COMPARATIVE COSTS FOR CHEMICALS

The experiments showed that small amounts of ethylene dichloride and carbon tetrachloride could be substituted successfully for a part of the requirement for ethylene chlorohydrin. Since at present prices the dichloride and tetrachloride are less expensive than chlorohydrin, the cost for the combined chemical is found to be much less than that for chlorohydrin alone. On the basis that 0.4 cc. of "rindite" is equivalent to 0.8 cc. of ethylene chlorohydrin (per kg. of tubers), which according to Figure 1 would be a conservative estimate, the cost of the chemicals needed to treat 100 pounds of tubers can be computed. Assuming that the approximate prices are: ethylene chlorohydrin (anhydrous), \$1.00 per pound; ethylene dichloride, 8.42 cents per pound; carbon tetrachloride, 73 cents per gallon; and on the basis that the specific gravities of the liquids listed in the above order are: 1.205, 1.2455, and 1.595, the costs of the chemicals for treating 100 pounds of tubers are: ethylene chlorohydrin alone, 9.57 cents, the combined chemicals as "rindite," 3.20 cents.

#### SUMMARY

Although ethylene dichloride ( $\text{CH}_2\text{ClCH}_2\text{Cl}$ ) and carbon tetrachloride ( $\text{CCl}_4$ ) are much less effective than ethylene chlorohydrin ( $\text{CH}_2\text{ClCH}_2\text{OH}$ ) in hastening the germination of dormant potato (*Solanum tuberosum* L.) tubers they produce an effect at a much lower concentration. An effective combination of these three chemicals was obtained by mixing seven parts

by volume of ethylene chlorohydrin, three parts of ethylene dichloride and one part of carbon tetrachloride. The combined chemical was given the name "rindite," a word formed by taking one syllable from each of the constituents.

Potato tubers of three different varieties, two sizes, and at intervals after harvest were treated with varying concentrations of "rindite" and of each of the three constituents separately, and the average time required for the emergence of sprouts for each chemical in each lot was noted.

Graphs were prepared showing the relation between the amount of each chemical used and the germination response. Since there was no volume change on mixing the three components the amount of each component which was taken when a given amount of "rindite" was used could be calculated. Interpolations on the graphs demonstrated that the gain in germination time due to "rindite" was greater than the sum of the gains due to the action of the separate components. Two different methods of obtaining the sum of the effects of the separate components were used.

Data from the nine different tests were combined by expressing each germination time as a per cent of the control for that test, and averaging the results for each chemical. An empirical equation, which allowed computing the average germination time for each of the concentrations of chemical, was obtained. Gains due to "rindite" and to each of the three components as calculated from these equations showed a greater gain with the amount of "rindite" used than that due to the sum of the gains obtained with the corresponding amounts of the three separate components.

There was obtained some evidence that this extra gain due to "rindite" over that expected from the effect of the components taken separately may be due to an effect of ethylene chlorohydrin in increasing the permeability of the intact tubers to ethylene dichloride and carbon tetrachloride.

If the definition of synergism given in the dictionary as "cooperative action of discrete agencies such that the total effect is greater than the sum of the two effects taken independently" is accepted, the results of the experiments, as a whole, support the view that synergistic effects with these chemicals were obtained.

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## FURTHER TESTS OF THE USE OF THE METHYL ESTER OF ALPHA-NAPHTHALENEACETIC ACID FOR INHIBITING THE SPROUTING OF POTATO TUBERS

F. E. DENNY

Previous experiments (1, 2) have shown the effectiveness of the vapor of the methyl ester of alpha-naphthaleneacetic acid ( $C_{10}H_7CH_2COOCH_3$ ) in inhibiting the sprouting of the tubers of the potato (*Solanum tuberosum* L.), either at constant storage temperatures of 10°, 15°, 18°, and 22° C. (50°, 59°, 64.4°, and 71.6° F.), or in ventilated storage at a temperature which changed gradually from autumn through winter to spring within the range of the above series of temperatures.

The temperature of storage of potato tubers is important for determining their culinary quality, since it determines their reducing sugar content, and this in turn determines the color of the product when the tissue is cooked by frying (3). If the temperature is sufficiently low to prevent sprouting, say 5° to 6° C. (41° to 42.8° F.), an undesirable amount of reducing sugar accumulates, but if it is as high as 15° C. (59° F.) a considerable loss occurs by sprouting. This sprouting is, of course, prevented by the treatment with the methyl ester of alpha-naphthaleneacetic acid, but previous tests (2, p. 253) showed that at 15° C. there was some shrinkage of tubers due to moisture loss even though no sprouts developed, and at 10° C. (50° F.) some of the varieties showed considerable amounts of reducing sugar, although on prolonged storage the sugar content was reduced to low values (3, p. 299).

These results indicated that the most suitable storage temperature to avoid reducing sugar accumulation due to low temperature, and at the same time to avoid shrinkage due to high temperature, was in the range 10° to 15° C. (50° to 59° F.). The mid-point at 12.5° C. (54.5° F.) was selected for these experiments, and the object was to determine whether potato tubers stored over-winter at this temperature in the presence of the methyl ester of alpha-naphthaleneacetic acid would fail to sprout or shrink, and would furnish in the spring a stock of tubers with low reducing sugar content from which potato chips with a desirable color (3, p. 297) could be made.

The previous experiments on the use of different media as carriers in which to incorporate the methyl ester had suggested the use of shredded paper in the form of ribbon-like strips (1, p. 394). Later experiments



showed that good distribution of vapor among the tubers undergoing treatment was obtained in this way. The present experiments deal with the question as to the amount of paper needed in proportion to the weight of tubers treated.

By the use of talcum powder as a carrier (1, p. 393), it was found that amounts of the methyl ester as low as 25 mg. per kg. of tubers were effective. Tubers treated with talcum, however, are rather unsightly, and in the present experiments garden soil was tried as a substitute for talcum.

The results have shown in general that: 1. at a temperature of 12.5° C. (54.5° F.) tubers were stored from October 19 and 20 until April 2 without occurrence of sprouting and without undue shrinkage by loss of moisture, and that such tubers contained only traces of reducing sugar and furnished potato chips of good color; 2. the amount of shredded paper in which the methyl ester is incorporated need not be greater than one-sixteenth pound per bushel of tubers; 3. the chemical may be incorporated into either talcum or garden soil and applied as a dust, but that the amount of chemical may not be reduced much below 25 mg. of the methyl ester per kg. of tubers.

## RESULTS

### TREATMENT OF TUBERS IN GLAZED EARTHENWARE JARS

*Chemical incorporated into paper.* Tubers of the varieties Warba, Green Mountain, Irish Cobbler, and Katahdin were used in this experiment and were stored in two-gallon earthenware jars, nine pounds of tubers per jar, three jars for each treatment. The chemical was incorporated into shredded newspaper in the form of ribbon-like strips approximately 0.25 inch wide, 17 grams of paper being used per jar (approximately 0.25 lb. of paper per bushel of tubers). The required amount of the chemical, methyl ester of alpha-naphthaleneacetic acid, was dissolved in acetone which was then distributed evenly throughout the paper by means of a pipette, there being several successive applications at intervals, with rearrangement of the paper after each application. The amount of the methyl ester was varied so as to furnish the chemical in the graded series: 100 mg., 50 mg., and 25 mg. per kg. of the tubers to be treated. The control lots received a similar amount of paper and acetone, and were handled in the same way except that no chemical was dissolved in the acetone. After the acetone had evaporated, the shredded paper was distributed evenly among the tubers, and the jar was covered with wrapping paper in which five holes were punched with a pencil. All lots were stored in a constant temperature room maintained at 12.5° C. (54.5° F.).

The storage was started October 19, 1944, and was ended April 2, 1945. The results with the varieties Irish Cobbler and Warba are shown in Figure 1 A and B, and the results with Green Mountain and Katahdin

(not shown here) were similar. The treatments with 100 mg. of methyl ester per kg. of tubers inhibited sprout development almost completely, there being only a few of the tubers showing any sprouts, and these being only 2 to 3 mm. long. The lots treated with 50 mg. of methyl ester per kg. of tubers showed small sprouts on nearly all of the tubers, but it is not believed that this amount of sprout development should be regarded as excessive. Lots receiving only 25 mg. per kg. showed sprouts up to 2 inches long.

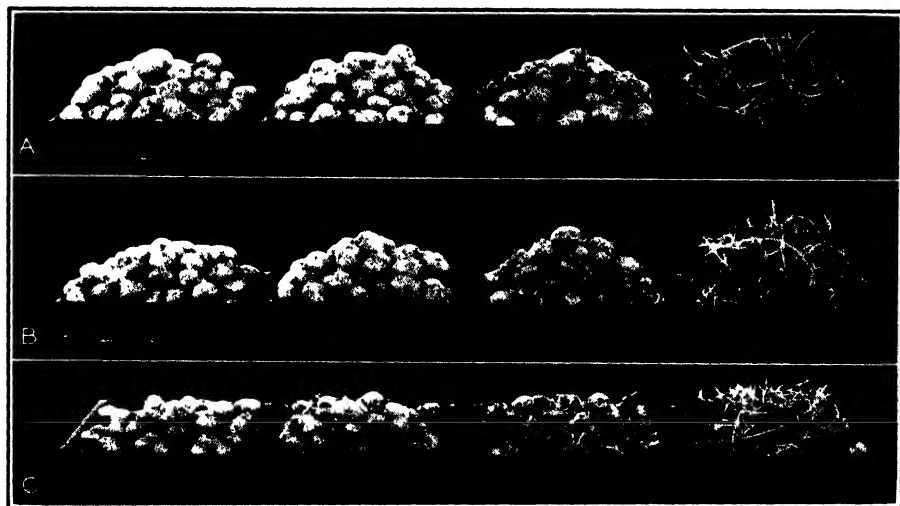


FIGURE 1. Inhibition of sprouting of potato tubers by methyl ester of  $\alpha$ -naphthaleneacetic acid. Storage temperature, 12.5° C. (54.5° F.) starting Oct. 19 and 20, 1944, ending April 2, 1945. A and B, treated with methyl ester incorporated into shredded paper; C, treated with methyl ester incorporated into garden soil and applied as a dust. Concentrations of methyl ester were: left to right in A and B, 100 mg. per kg. of tubers, 50 mg., 25 mg., control; left to right in C, 45 mg. per kg. of tubers, 15 mg., 5 mg., control. Varieties: A, Irish Cobbler; B, Warba; C, Katahdin.

The photographs do not show adequately the firmness of the tubers, but the tubers were examined for this characteristic, and it may be stated that all lots receiving either 100 mg. or 50 mg. of methyl ester per kg. of tubers were in good condition. There were small differences among the varieties, and the firmness was in the order Irish Cobbler, Green Mountain, Katahdin, and Warba, the Warba tubers being the least firm. Probably potato tubers should not be less firm than the Warba tubers in this test. This indicates that for over-winter storage the temperature should not be much higher than 12.5° C. (54.5° F.), although of course the type of container, bulk of the product, humidity of the air, amount of ventilation, etc., would be important factors in practice.

*Chemical incorporated into powder.* Tubers of the varieties Irish Cobbler and Katahdin were used in this test. Both talcum powder and soil were used as carriers. The soil was air-dry garden soil powdered to pass through a 60-mesh sieve. The amount of powder (either talcum or soil) used was adjusted on the basis that, as shown by a preliminary test, 1.65 g. of powder adhered to each kg. of potato tubers when the powder was applied by hand to each tuber, by rubbing the tuber and then tapping it lightly. The required amount of methyl ester was dissolved in acetone and the acetone solution was added to the powder. After the evaporation of the solvent, the powder was dried thoroughly and ground with a mortar and pestle. The amount of methyl ester was adjusted to give the series: 45 mg., 15 mg., and 5 mg. of the chemical per kg. of tubers treated. The control tubers were handled in the same way except that the acetone applied to the powder contained no methyl ester. The dusted tubers were placed in two-gallon earthenware jars, two jars per lot, and were covered with paper in which five holes were punched with a pencil. All lots were stored at 12.5° C. (54.5° F.) from October 20, 1944 to April 2, 1945.

Figure 1 C shows the results with the Katahdin variety treated with methyl ester incorporated into soil and the results with Irish Cobbler were similar in all respects. Also, the lots treated with the methyl ester incorporated into talcum powder were quite similar to those shown for garden soil, although of course they were white in color, due to the presence of the talcum.

Tubers treated with 45 mg. of methyl ester per kg. were in firm condition and without sprouts. The amount of sprouting and shrinkage of the tubers increased with the decrease in the amount of methyl ester used. The lots receiving 15 mg. per kg. were below the limits both from the standpoint of sprouting and firmness. Probably the lower limit with treatments with either talcum or soil as carriers is 25 mg. of methyl ester per kg. of tubers, which is the amount found satisfactory in the previous experiment (1, p. 394).

#### TREATMENT OF TUBERS IN WOODEN BOXES

The object of this test was to obtain data on the amount of shredded paper treated with the methyl ester of alpha-naphthaleneacetic acid needed in proportion to the amount of potato tubers treated. The amount of methyl ester used was the same for each lot, i.e., 100 mg. of methyl ester per kg. of tubers, but this amount was incorporated into different amounts of shredded paper to give the series one-fourth, one-eighth, and one-sixteenth pound of paper per bushel of potato tubers. The storage containers used were wooden boxes, of a size to contain 60 lb. for tubers of the variety Irish Cobbler, and 36 lb. for those of Katahdin. The boxes were lined with paper on all sides, and were covered with wooden slats. The required amount of shredded paper to give the proportions indicated above were

distributed evenly among the tubers. These containers were placed in the storage room at 12.5° C. (54.5° F.) on October 20, 1944, and the experiment was discontinued April 2, 1945.

There was definitely more sprouting among the lots in these containers than in those stored in the earthenware jars. Probably the volume of tubers was too small for preventing dissipation of the vapor through the paper and wooden walls. However, the conditions should be sufficient to furnish comparisons as to the effectiveness of different amounts of paper. At the end of the storage period, the sprouts were removed from all tubers and were weighed. The results are shown in Table I. The experiment failed to show definitely the lower limits of the quantity of paper needed, the results with one-sixteenth pound of paper per bushel of tubers not differing significantly from those in which one-fourth pound was used. The amount of sprouting was not sufficiently large to cause serious shrinkage of the tubers, the tubers of the treated lots being in a satisfactory condition as to firmness.

TABLE I  
EFFECT OF AMOUNT OF SHREDDED PAPER IMPREGNATED WITH METHYL ESTER OF  
ALPHA-NAPHTHALENEACETIC ACID USED IN TREATING POTATO TUBERS

Amount of shredded paper per bushel of tubers	Amount of methyl ester per kg. of tubers, mg.	Weight of sprouts (oz.)	
		Per 60 lb. of Irish Cobbler potatoes	Per 36 lb. of Katahdin potatoes
1/4 lb.	100*	2.54	0.92
1/8 lb.	100	1.27	1.21
1/16 lb.	100	2.15	1.66
1/4 lb.	0	40.73	31.92

\* Equivalent to 0.006 oz. per bushel.

#### SUGAR CONTENT AND POTATO CHIP COLOR

As soon as the potatoes were removed from the storage room on April 2, 1945, tuber samples were removed from the lots that had received either 100 mg. or 50 mg. of methyl ester incorporated into paper, or 45 mg. applied in the form of soil. Slices were cut for the preparation of potato chips and juice was squeezed for the sugar analyses, the methods used being those previously described (3, p. 293, 294). Only traces of reducing sugar were found in the juice of each lot, and the potato chips obtained were of good color (3, p. 297, see Lot 2 A).

#### SUMMARY

Tests were made of the suitability of a temperature of 12.5° C. (54.5° F.) for storage of potato tubers undergoing treatment with the methyl ester of alpha-naphthaleneacetic acid ( $C_{10}H_7CH_2COOCH_3$ ) to inhibit the sprouting and to maintain the tubers in firm condition without excessive shrinkage during over-winter storage.

Tubers of four varieties of potato (*Solanum tuberosum* L.) were stored at 12.5° C. (54.5° F.) from October 19 and 20, 1944 to April 2, 1945 in the presence of different concentrations of the vapor of methyl ester of  $\alpha$ -naphthaleneacetic acid.

When the treatments were applied by impregnating narrow strips of paper with the chemical and distributing the shredded paper among the tubers, sprouting was inhibited by the use of 100 mg. of chemical per kg. of tubers, and nearly so by 50 mg., but at 25 mg. considerable sprout development occurred.

When 100 mg. of the chemical per kg. of tubers was incorporated into varying amounts of shredded paper, it was found that the amount of paper could be varied at least over the range one-fourth to one-sixteenth pound of paper per bushel of tubers treated.

When the treatments were applied by incorporating the chemical into either talcum powder or garden soil sifted to pass through a 60-mesh sieve, sprouting was inhibited by the use of 45 mg. of methyl ester per kg. of tubers, but not by the use of 15 mg. or less. Previous results had shown that 25 mg. per kg. could be used successfully.

The tubers of lots treated with methyl ester at the rate of 100 mg. or 50 mg. per kg. in shredded paper, or with 45 mg. in talcum powder or garden soil, were in firm condition without excessive shrinkage. There was some evidence that for over-winter storage the temperature should not be much higher than that used in these tests, 12.5° C. (54.5° F.).

At the end of the experiment, samples of tubers showed that only traces of reducing sugar were present in the juice, and slices of the tubers furnished potato chips of good color.

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# METHODS OF RATING THE ROOT-INDUCING ACTIVITY OF PHENOXY ACIDS AND OTHER GROWTH SUBSTANCES

A. E. HITCHCOCK AND P. W. ZIMMERMAN

Considering the relatively high activity shown by some of the substituted phenoxy compounds in earlier tests, it seemed of interest to test all phenoxy compounds available at that time. Accordingly, in 1943 this was done, using 63 phenoxy compounds together with naphthaleneacetic and indolebutyric acids. The results of these tests, for which only a brief summary was given before (4), are recorded in the present report. Eighteen of the 63 used in 1943 were selected for testing again in 1944. The results of these later tests constitute the principal part of the present paper. The relation between the structure of certain compounds and root-inducing activity has been discussed in previous reports (2, 3, 4).

In most of the tests relating to root-inducing activity comparative differences have been based on the average number of roots per cutting. This method appeared to be satisfactory for a small number of compounds, but with larger numbers of compounds the differences became smaller and the relative order of activity was not always the same in different tests. It thus appeared desirable to determine by statistical analysis which compounds were essentially alike and which ones were significantly different.

Two methods were used—namely, the analysis of variance and the method of ranks devised by Friedman (1). From the standpoint of ranking the acids on the basis of the average number of roots for 12 treatments, the two methods gave essentially the same results. The two methods showed agreement also in classifying concentration and the type of cutting according to the acids used in each series of tests. Though not of recent origin, the method of ranks should prove useful to some workers, particularly in view of the fact that it may be used without a detailed knowledge of statistical procedures.

The active phenoxy acids fell into three categories. 2,4,5-Trichlorophenoxyacetic acid and  $\alpha$ -(2,4,5-trichlorophenoxy)-propionic acid were of approximately equal activity to  $\alpha$ -naphthaleneacetic acid for inducing normal roots.  $\alpha$ -(2,4-Dichlorophenoxy)-propionic,  $\alpha$ -(2,4,5-trichlorophenoxy)-*n*-butyric, and  $\alpha$ -(2,4-dichlorophenoxy)-*n*-butyric acids were less active than  $\alpha$ -naphthaleneacetic acid. The monochlorophenoxy acids and  $\alpha$ -(2,5-dimethylphenoxy)-propionic acid were least active. This is in

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general agreement with the results of the 1943 tests with one exception. The relative activities of  $\alpha$ -(2,4-dichlorophenoxy)-propionic and 2,4,5-trichlorophenoxyacetic acids were reversed in the 1944 tests, the latter acid being the most active.

### MATERIALS AND METHODS

Privet (*Ligustrum ovalifolium* Hassk.) shoots collected three different times during July, 1944, are subsequently referred to as series A, B, and C. In each series the shoots were segregated into four types (I, II, III,

TABLE I  
CODE NAMES ASSIGNED TO ACIDS TOGETHER WITH THE CONCENTRATIONS  
USED IN EACH SERIES OF TESTS

Code name	Name of acid	Concn. mg./l.	Series
2-CIPOA	2-Chlorophenoxyacetic	0.32, 1.0, 3.2, 10	C
2-CIPOP	$\alpha$ -(2-Chlorophenoxy)-propionic	"	A, C
2-CIPOB	$\alpha$ -(2-Chlorophenoxy)- <i>n</i> -butyric	"	C
3-CIPOA	3-Chlorophenoxyacetic	"	C
3-CIPOP	$\alpha$ -(3-Chlorophenoxy)-propionic	"	A, C
3-CIPOB	$\alpha$ -(3-Chlorophenoxy)- <i>n</i> -butyric	"	C
4-CIPOA	4-Chlorophenoxyacetic	"	C
4-CIPOP	$\alpha$ -(4-Chlorophenoxy)-propionic	"	A, C
4-CIPOB	$\alpha$ -(4-Chlorophenoxy)- <i>n</i> -butyric	"	C
Cl <sub>2</sub> POA	2,4-Dichlorophenoxyacetic	0.1, 0.32, 1.0, 3.2	B
Cl <sub>2</sub> POP	$\alpha$ -(2,4-Dichlorophenoxy)-propionic	"	A, B
Cl <sub>2</sub> POB	$\alpha$ -(2,4-Dichlorophenoxy)- <i>n</i> -butyric	"	B
(CH <sub>3</sub> ) <sub>2</sub> POA	2,5-Dimethylphenoxyacetic	0.32, 1.0, 3.2, 10	B
(CH <sub>3</sub> ) <sub>2</sub> POP	$\alpha$ -(2,5-Dimethylphenoxy)-propionic	"	A, B
(CH <sub>3</sub> ) <sub>2</sub> POB	$\alpha$ -(2,5-Dimethylphenoxy)- <i>n</i> -butyric	"	B
Cl <sub>3</sub> POA	2,4,5-Trichlorophenoxyacetic	0.1, 0.32, 1.0, 3.2	B
Cl <sub>3</sub> POP	$\alpha$ -(2,4,5-Trichlorophenoxy)-propionic	"	A, B
Cl <sub>3</sub> POB	$\alpha$ -(2,4,5-Trichlorophenoxy)- <i>n</i> -butyric	"	B
NA	$\alpha$ -Naphthalenacetic	3.2, 10, 20, 32	A, B, C
IB	$\beta$ -Indolebutyric	10, 32, 48, 64	A, B, C

and IV) according to leaf and stem characteristics and to their location on the parent plants. There was no direct relation between the types in the three series. For example, type II was not the same in series C as in series A. The treatments used are shown in Table I. Each concentration was applied to three cuttings of each of the four types, or a total of 12 cuttings. The average number of roots on these 12 cuttings was used for each point on the curves in Figures 1, 2, and 3.

The cuttings were treated according to the standard procedure by immersing the basal ends in the test solution for a period of 20 hours in the laboratory. After this they were planted in sand in greenhouse benches. Root counts were made 25 days after treatment in the case of series A and B, and after 29 days in the case of series C. Root counts were made when control cuttings started to root.

Most of the phenoxy compounds used in the 1943 and 1944 tests were synthesized in the laboratories of the Boyce Thompson Institute. The code names for the acids and the concentrations used in the 1944 tests appear in Table I.

#### STATISTICAL METHODS

Data selected for statistical analysis apply only to those acids which induced an average number of roots greater than that for any of the tap

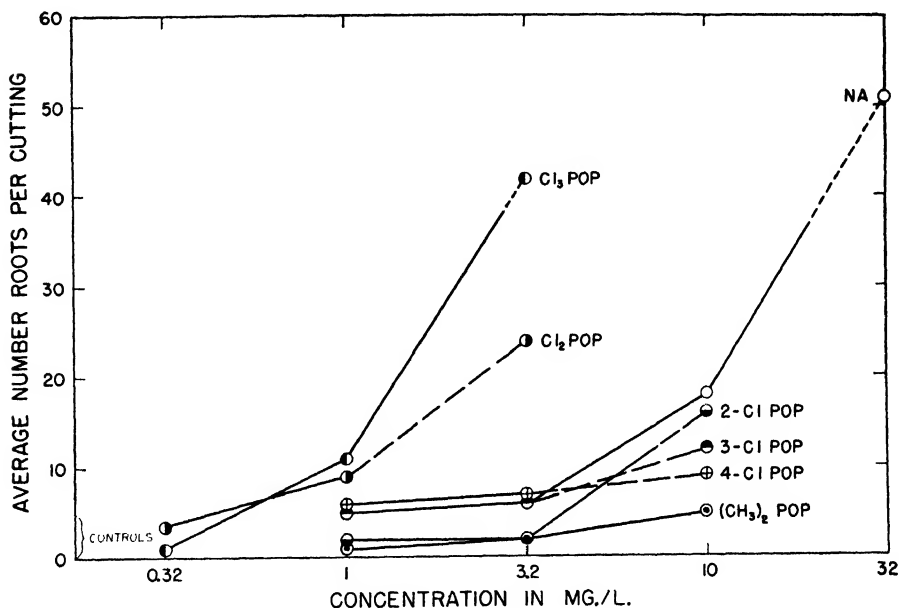


FIGURE 1. Comparative activity of acids used in series A. Relative length of broken lines represents the relative degree of overtreatment at the higher concentration. Short dashes represent slight overtreatment and long dashes noticeable overtreatment.

water controls. Curves in Figures 1, 2, and 3 show which acids were inactive for inducing roots in those tests. A further selection was made on the basis of concentration effects. Three of the four concentrations selected from each series represent the ascending portion of the root count curves which were not associated with excessive abnormalities in rooting (Figs. 1, 2, and 3). For the monochlorophenoxy acids this range consisted of the three highest concentrations in the case of series A and of the three lowest in series C. Indolebutyric acid was omitted because there were not three concentrations having an interval of 3.2 times as in the case of all other acids. The 12 treatments used as a basis of comparison consisted of three concentrations applied to each of four types of cuttings. The 12 individual



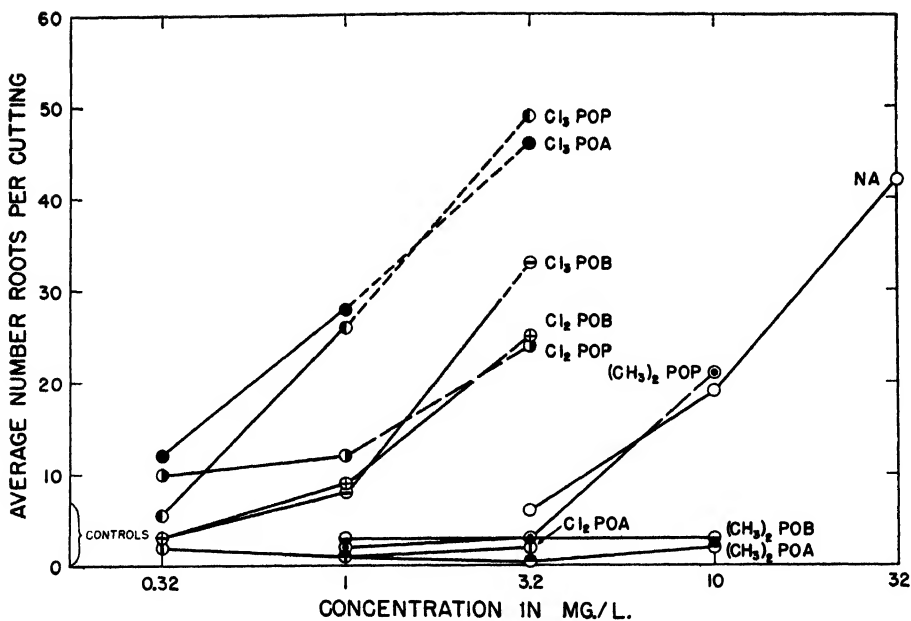


FIGURE 2. Comparative activity of acids used in series B. Relative length of broken lines represents the relative degree of overtreatment at the higher concentration. Short dashes represent slight overtreatment and long dashes noticeable overtreatment.

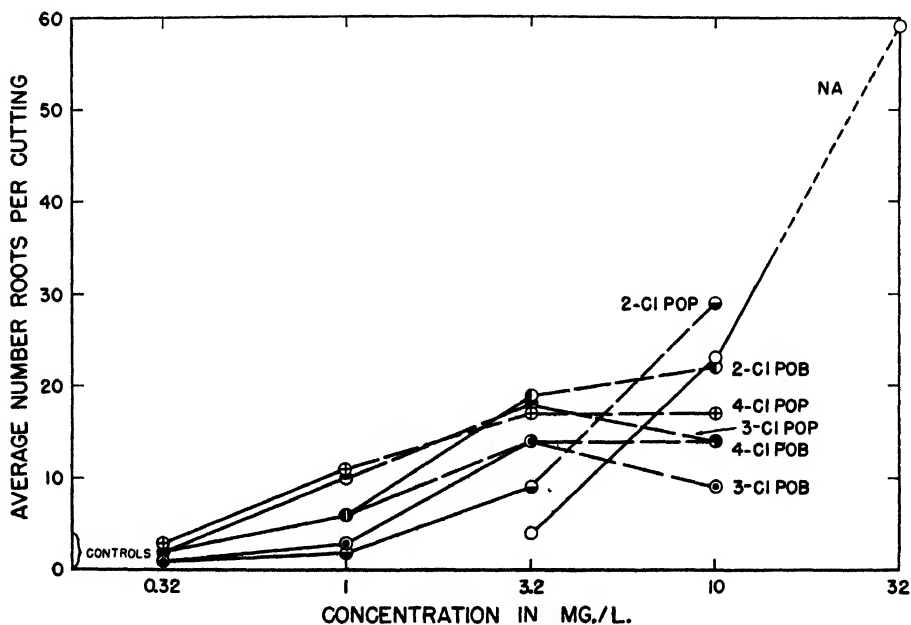


FIGURE 3. Comparative activity of acids used in series C. Relative length of broken lines represents the relative degree of overtreatment at the higher concentration. Short dashes represent slight overtreatment and long dashes noticeable overtreatment.

root count values for each of the seven acids in Table III are the averages for three cuttings. Since 84 treatments were used in each of the three series (Table III), the total degrees of freedom are the same, namely 83. The error term for the analysis of variance is likewise the same (acid  $\times$  concentration  $\times$  type) with a  $t$  value of 2.030 for 36 degrees of freedom. Procedures used in the method of ranks appear in Table II and are further described in the text under results.

TABLE II  
PROCEDURES USED FOR OBTAINING  $\chi_r^2$  VALUE BY THE METHOD OF RANKS

Root counts from series A							Ranked root counts						
NA	Cl <sub>3</sub> POP	Cl <sub>2</sub> POP	3-Cl POP	4-Cl POP	2-Cl POP	(CH <sub>3</sub> ) <sub>2</sub> POP	NA	Cl <sub>3</sub> POP	Cl <sub>2</sub> POP	3-Cl POP	4-Cl POP	2-Cl POP	(CH <sub>3</sub> ) <sub>2</sub> POP
13	0	5	6	0	0	2	1	6	3	2	6	6	4
17	17	10	1	1	3	3	1.5	1.5	3	6.5	6.5	4.5	4.5
58	33	34	13	14	4	10	1	3	2	5	4	7	6
2	3	2	1	14	2	0	4	2	4	6	1	4	7
21	9	13	11	8	0	2	1	4	2	3	5	7	6
53	51	29	11	5	13	0	1	2	3	5	6	4	7
0	1	0	10	4	0	0	5.5	3	5.5	1	2	5.5	5.5
16	5	8	3	8	1	0	1	4	2.5	5	2.5	0	7
38	35	15	5	5	35	1	1	2.5	4	5.5	5.5	2.5	7
8	0	10	3	6	2	1	2	7	1	4	3	5	6
18	13	4	10	12	3	2	1	2	5	4	3	6	7
56	51	14	21	10	12	10	1	2	4	3	6.5	5	6.5
300	218	149	95	87	75	31	21	39	39	50	51	66.5	73.5

#### Calculations

$$\chi_r^2 = \frac{12}{np(p+1)} \times \Sigma(\text{column totals})^2 - 3n(p+1)$$

In which  $n$  = number of sets of ranks = 12

$p$  = number of ranks = 7

$$\Sigma(\text{Column totals})^2 = 21^2 + 39^2 + 39^2 + 50^2 + 51^2 + 66.5^2 + 73.5^2 = 18,408.5$$

$$\chi_r^2 = \frac{12}{12 \times 7 \times 8} \times 18,408.5 - 3 \times 12 \times 8 = 40.723.$$

The  $n$  value to be used in the  $\chi^2$  table (6) is one less than the number of ranks ( $p-1$ ) = 6. For  $n=6$  and  $P.01$ ,  $\chi^2 = 16.812$ .

$\therefore \chi_r^2 = 40.732 > P.01$ , showing the seven acids to be unlike for inducing roots.

## EXPERIMENTAL RESULTS

### RESULTS OF 1943 TESTS

All of the compounds used in the 1943 tests now appear for the first time. They have been arranged in each group in descending order according to the average number of roots (for three concentrations) induced on privet cuttings. With few exceptions (indicated by an asterisk) there was no replication of treatments. Some of the relations between structure and root-inducing activity were discussed briefly in a previous report (4).

TABLE III  
AVERAGE NUMBER OF ROOTS ON THREE CUTTINGS

Code name of acid	Type I			Type II			Type III			Type IV			Line totals
	L	M	H	L	M	H	L	M	H	L	M	H	
Series A† (L.S.D.† = 62)													
NA	13	17	58	2	21	53	0	16	38	8	18	56	300
Cl <sub>3</sub> POP	0	17	33	3	9	51	1	5	35	0	13	51	218
Cl <sub>2</sub> POP	5	10	34	2	13	29	0	8	15	10	4	19	149
3-CIPOP	0	1	13	1	11	11	10	3	5	3	10	21	95
4-CIPOP	0	1	14	14	8	5	4	8	5	6	12	10	87
2-CIPOP	0	3	4	2	0	13	0	1	35	2	3	12	75
(CH <sub>3</sub> ) <sub>2</sub> POP	2	3	10	0	2	0	0	0	1	1	2	10	31
Series B† (L.S.D. = 74)													
Cl <sub>3</sub> POA	7	13	62	21	20	33	12	43	45	8	35	44	343
Cl <sub>2</sub> POP	4	21	52	3	26	23	11	32	59	9	25	63	328
NA	4	16	43	3	21	28	9	32	44	7	9	52	268
Cl <sub>2</sub> POP	3	5	25	11	8	15	8	21	26	17	14	29	182
Cl <sub>2</sub> POB	0	5	27	3	5	29	7	15	36	3	9	39	178
Cl <sub>2</sub> POB	1	10	19	5	3	9	4	14	36	2	9	36	148
(CH <sub>3</sub> ) <sub>2</sub> POP	0	0	9	1	0	15	2	5	24	6	8	37	107
Series C†† (L.S.D. = 56)													
NA	3	34	51	1	3	67	3	18	62	10	36	57	345
4-CIPOP	1	9	1	1	5	18	0	16	24	9	15	25	124
3-CIPOP	0	10	16	3	5	17	1	3	12	3	24	29	123
2-CIPOB	1	2	15	2	9	16	2	1	16	3	11	27	105
4-CIPOB	3	3	10	0	1	14	1	7	15	5	12	17	88
3-CIPOB	0	0	17	1	0	6	0	6	16	2	8	16	72
2-CIPOP	0	0	1	0	0	8	0	0	0	4	7	27	47
Analysis of Variance													
Factor	D.F.	Series A		Series B		Series C							
		Var.	F	Var.	F	Var.	F						
Acid	6	731	19.3**	691	12.4**	819	25.7**						
Concentration	2	2926	77.3**	5783	103.9**	2698	84.7**						
Type	3	57	1.5	714	12.8**	317	10.0**						
Acid X concentration	12	331	8.8**	116	2.1*	272	8.5**						
Acid X concentration X type													
(error)	36	38		56		32							
Residue	24	42				71							
Total	83												

† L, M, and H represent three highest concentrations (see Table I).

†† L, M, and H represent three lowest concentrations (see Table I).

\* Significant.

\*\* Highly significant.

‡ All L.S.D. values at 5 per cent level.

Only a few of these phenoxy compounds were listed in the report just mentioned.

*Group 1.* Compounds which induced a relatively large number of roots over a fairly wide range of concentrations. Most phenoxy acids induced the formation of some fasciated or large diameter roots at near optimal concentrations but caused little or no stem injury.

	Average number of roots
$\alpha$ -(2,4,5-Trichlorophenoxy)-propionic acid*	27
$\alpha$ -Naphthaleneacetic acid*	26
$\alpha$ -(2,4-Dichlorophenoxy)-propionic acid*	24
$\alpha$ -(3-Chlorophenoxy)- <i>n</i> -butyric acid	21
$\alpha$ -(2,4-Dibromophenoxy)-propionic acid	21
2,4,5-Trichlorophenoxyacetic acid*	18
$\alpha$ -(2-Chlorophenoxy)- <i>n</i> -butyric acid	14
$\beta$ -Indolebutyric acid*	14

*Group 2.* Compounds which induced a moderately large number of roots over a relatively narrow range of concentrations. Generally abnormal roots or stem injury occurred at near optimal concentrations for number of roots.

	Average number of roots
$\alpha$ -(2,4,5-Trichlorophenoxy)- <i>n</i> -butyric acid	14
$\alpha$ -(2,4-Dichlorophenoxy)- <i>n</i> -butyric acid*	12
$\alpha$ -(3-Chlorophenoxy)-propionic acid	10
$\alpha$ -(2,5-Dimethylphenoxy)-propionic acid	9
$\alpha$ -(2-Methylphenoxy)- <i>n</i> -butyric acid	9
$\alpha$ -(Phenoxy)-butyric acid	9
$\alpha$ -(4-Chlorophenoxy)-propionic acid*	8
$\alpha$ -(4-Chlorophenoxy)- <i>n</i> -butyric acid*	7
$\alpha$ -(2-Methylphenoxy)-propionic acid	7
$\alpha$ -(2,5-Dimethylphenoxy)- <i>n</i> -butyric acid	7
$\alpha$ -(2-Chlorophenoxy)-propionic acid	5
$\alpha$ -(2,4-Dibromophenoxy)- <i>n</i> -butyric acid	4
4-Aminophenoxyacetic acid	4

*Group 3.* Compounds which induced a larger number of roots than the water controls, but resulted in no well defined concentration effect except as associated with abnormal roots or injury to the stem.

	Average number of roots
$\alpha$ -(Phenoxy)-propionic acid	5
4-Methylphenoxyacetic acid	5
2,4-Dichlorophenoxyacetic acid*	4
2,4,6-Triiodophenoxyacetic acid	4

	Average number of roots
2-Iodophenoxyacetic acid	3
2-Nitrophenoxyacetic acid	3
3-Nitrophenoxyacetic acid	3
$\alpha$ -(2,4-Dichlorophenoxy)-isovaleric acid	2
2,4-Diiodophenoxyacetic acid	2
2,6-Dibromo-4-aminophenoxyacetic acid	2
Potassium-2,6-diiodo-4-carboxyphenoxyacetate	2
2-Methylphenoxyacetic acid	2
$\alpha$ -(4-Methylphenoxy)-propionic acid	2
$\alpha$ -(4-Methylphenoxy)- <i>n</i> -butyric acid	2

*Group 4.* Compounds which exhibited no concentration effect, causing responses similar to control water solutions.

Phenoxyacetic acid

2-Chlorophenoxyacetic acid\*  
 2-Aminophenoxyacetic acid  
 3-Chlorophenoxyacetic acid\*  
 3-Aminophenoxyacetic acid  
 4-Chlorophenoxyacetic acid\*  
 4-Bromophenoxyacetic acid  
 2,4-Dibromophenoxyacetic acid  
 2,4-Dinitrophenoxyacetic acid  
 $\alpha$ -(2,4-Dichlorophenoxy)-malonic acid  
 $\alpha$ -(2,4-Dichlorophenoxy)-caproic acid  
 $\alpha$ -(2,4-Dichlorophenoxy)-lauric acid  
 Ethyl 2,4-dimethylphenoxyacetate  
 $\alpha$ -(2,4-Dimethylphenoxy)-propionic acid  
 $\alpha$ -(2,4-Dimethylphenoxy)-*n*-butyric acid  
 2,5-Dimethylphenoxyacetic acid  
 3,4-Dimethylphenoxyacetic acid  
 $\alpha$ -(3,4-Dimethylphenoxy)-propionic acid  
 $\alpha$ -(3,4-Dimethylphenoxy)-*n*-butyric acid  
 3,5-Dimethylphenoxyacetic acid  
 $\alpha$ -(3,5-Dimethylphenoxy)-propionic acid  
 $\alpha$ -(3,5-Dimethylphenoxy)-*n*-butyric acid  
 $\beta$ -(3,5-Dimethylphenoxy)-*n*-butyric acid  
 2,4,6-Trichlorophenoxyacetic acid  
 $\alpha$ -(2,4,6-Trichlorophenoxy)-propionic acid  
 $\alpha$ -(2,4,6-Trichlorophenoxy)-*n*-butyric acid  
 2,4,6-Tribromophenoxyacetic acid  
 4-Chloro-3,5-dimethylphenoxyacetic acid  
 2,3,4,6-Tetrachlorophenoxyacetic acid  
 2,3,4,5,6-Pentachlorophenoxyacetic acid

## RESULTS OF 1944 TESTS

*Results Not Statistically Analyzed*

The descending order of activity for all of the active acids appears in Table VI together with the order for these same acids found in the 1943 tests. Root counts were excluded in the present tests in those cases where abnormal rooting had occurred. For example, 3-ClPOB and 2-ClPOB received the relatively high rating in 1943 (Table VI) mainly on the basis of the high root counts for the 10 mg./l. concentration which induced an average of 38 and 21 roots respectively for these two acids. Since the relatively high root counts for 10 mg./l. shown in Figure 3 were omitted for all monochlorophenoxy acids, these particular acids are given a much lower rating in the 1944 tests. However, in this case it is 2-ClPOP which has been penalized the most. The overtreatment effects induced by 2-ClPOP at 10 mg./l. in series C are shown in Figure 7. The 10 mg./l. concentration in series A was considered comparable to the 3.2 mg./l. concentration in series C. For comparative purposes in the statistical analyses it was believed preferable to compare root counts in the optimal part of the concentration range in each series of tests.

Relative differences in rooting response for the eight acids in series A are illustrated in Figure 5 for one of the four types and for all four concentrations. The best root systems are on cuttings treated with IB, NA, and Cl<sub>3</sub>POP and the poorest on cuttings treated with 2-ClPOP, 3-ClPOP, 4-ClPOP, and (CH<sub>3</sub>)<sub>2</sub>POP. The rooting response of Cl<sub>2</sub>POP was intermediate. Control cuttings had just started to root. These differences are typical of those obtained with the other three types of cuttings, since in this series the four types did not differ significantly as they did in series B and C (see *F* value for type in Tables III and IV). In this same figure the inhibiting effect of the highest concentration is most evident for 3-ClPOP and 4-ClPOP. A more extreme case of this type of overtreatment appears in Figure 4 in which there is also shown the relatively narrow effective range of concentrations typical of the monochlorophenoxy acids.

The differences in root-inducing activity due to length of side chain are shown in Figure 6. Cl<sub>3</sub>POA was the only phenoxy acid with an acetic acid side chain which was active and in this case was highly active. Another example of the difference due to length of side chain is shown for 4-chlorophenoxy acids in Figure 4. The influence of the number of chlorine substituents is also shown in Figure 6 where it is seen that the activity increases with the number from one to three. However, there was little difference in the activity according to the position occupied by a single chlorine substituent in the ring.

In Figure 7 the variation in rooting response according to the type of cutting is shown for treatments duplicated at different times. Rooting of

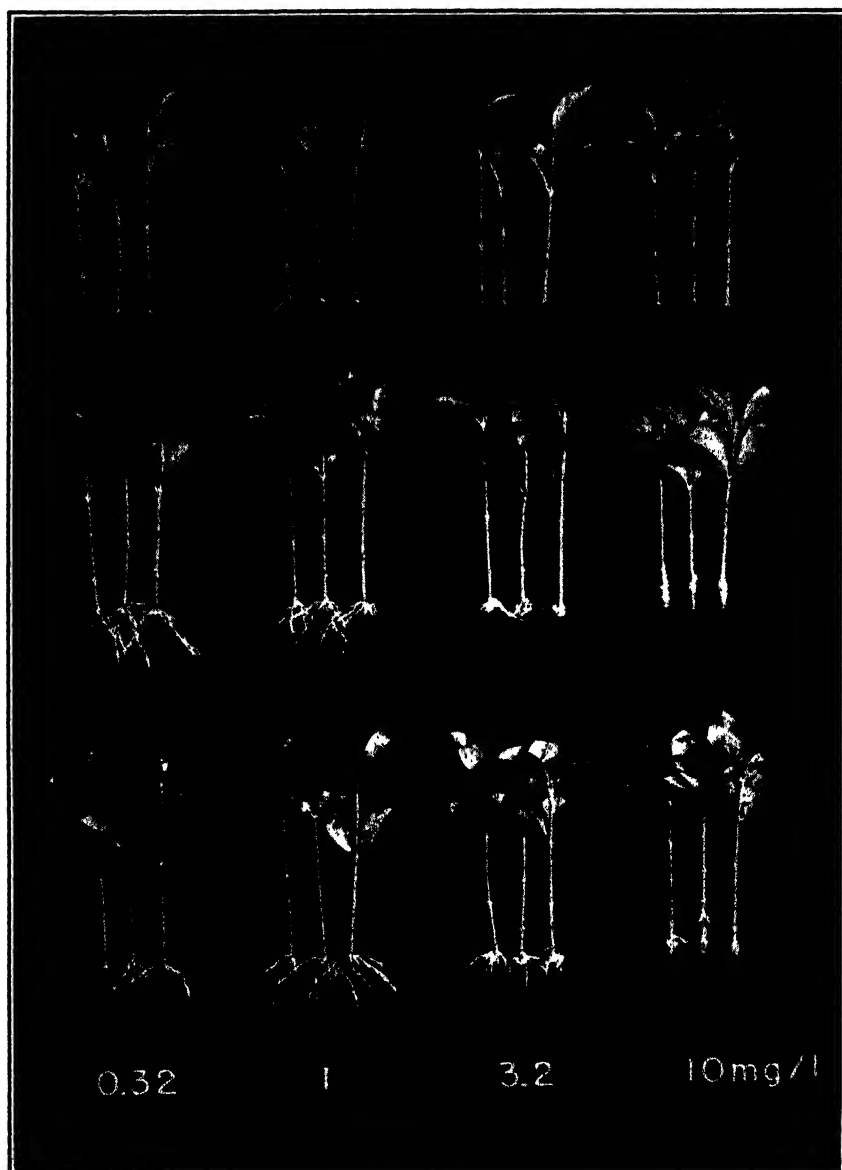


FIGURE 4. Privet cuttings 29 days after treatment (series C) showing influence of concentration, length of side chain, and relative position of cutting material on parent shoot. Upper row, 4-CIPOA; middle row, 4-CIPOP; and lower row, 4-CIPOB. In each set (left to right): tip, middle, and basal portion of same shoot. Excessive overtreatment and injury at 10 mg./l.

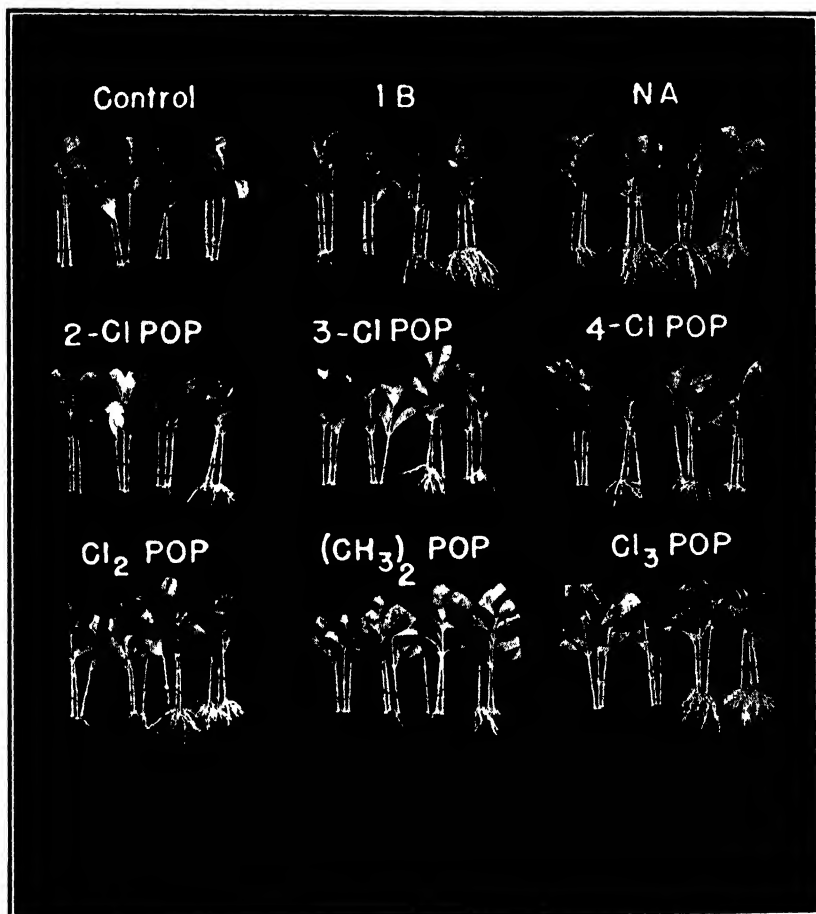


FIGURE 5. One of four types of privet cuttings used in series A, 25 days after treatment. Concentration in mg./l. (Left to right): for IB, 10, 32, 48, and 64; for NA, 3.2, 10, 20, and 32; for 2-ClPOP, 3-ClPOP, 4-ClPOP, and  $(\text{CH}_3)_2\text{POP}$ , 0.32, 1, 3.2, and 10; and for  $\text{Cl}_2\text{POP}$  and  $\text{Cl}_3\text{POP}$ , 0.1, 0.32, 1, and 3.2.

the four types is more uniform in series A than in series B or C. This was mainly due to the fact that in the first collection it was possible to obtain a larger number of comparable shoots than in later collections. The difference in the response of cuttings treated with the same acids at different times is most noticeable for 2-ClPOP, but this difference is seen to be due to a shifting of the optimal part of the concentration curve. Thus the response to a concentration of 3.2 mg./l. in series C was about comparable to 10 mg./l. in series A.

The results of all tests show that the four concentrations selected are



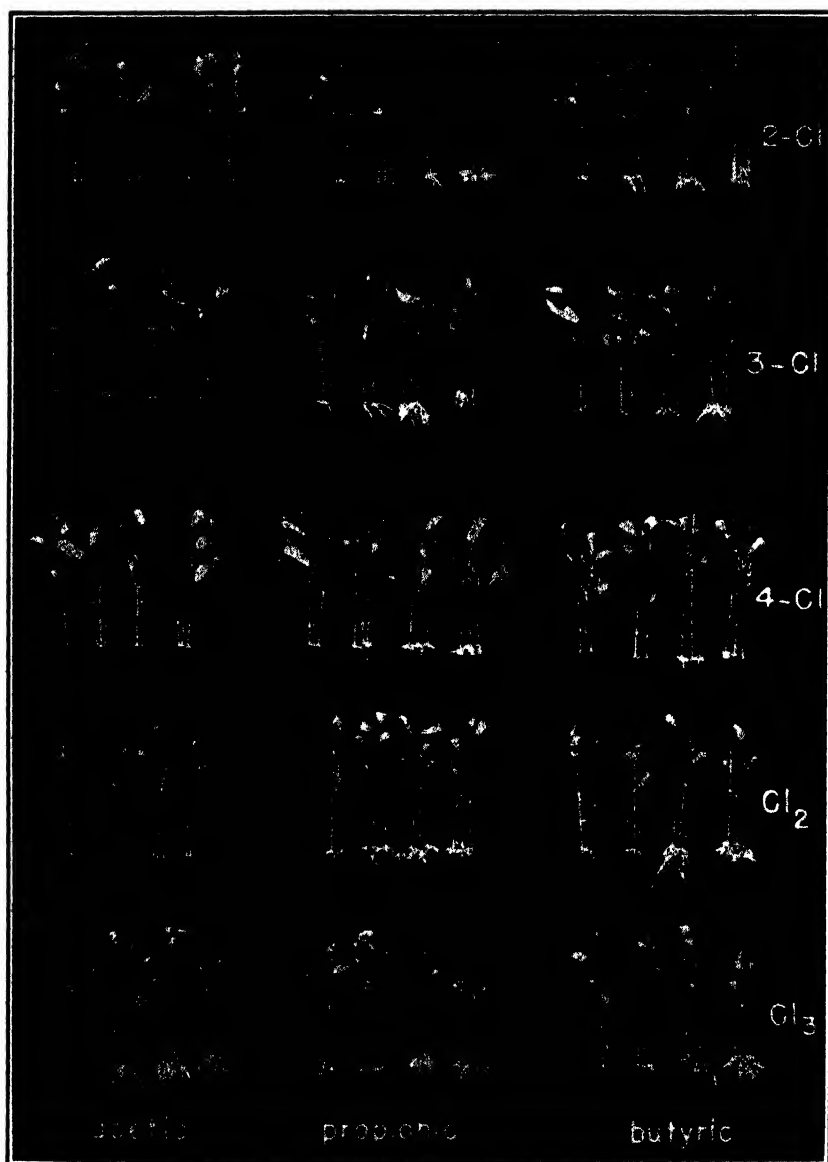


FIGURE 6. Influence of length of side chain, number of chlorine substituents in the ring, and the kind of acid on rooting of privet cuttings. Concentration in mg./l. in each group (left to right): for monochlorophenoxy acids in upper three rows, 0.32, 1, 3.2, and 10; for dichlorophenoxy and trichlorophenoxy acids, 0.1, 0.32, 1, and 3.2.

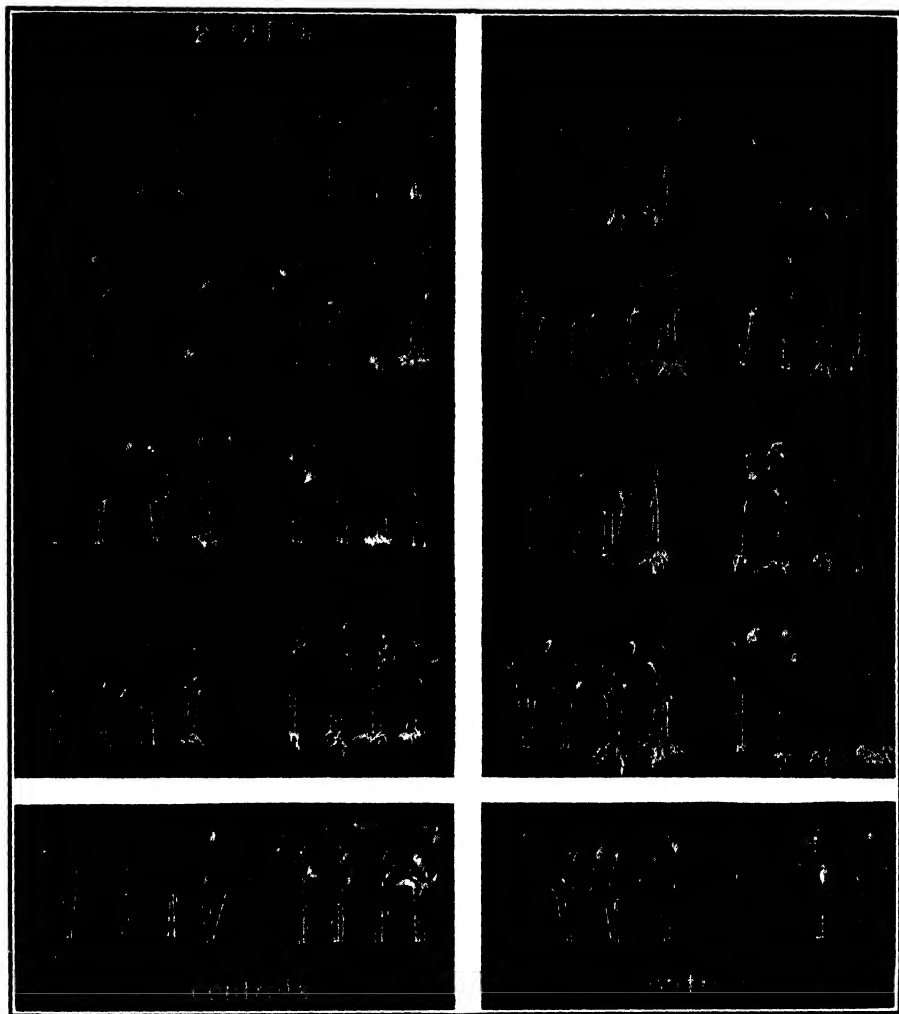


FIGURE 7. Variations due to duplication of tests at different times and to the use of four types of cuttings (I, II, III, and IV respectively from upper row). 2-ClPOP: column on left, series A; column on right, series C. Cl<sub>3</sub>POP: column on left, series A; column on right, series B. Concentration in each group (left to right): for 2-ClPOP, 0.3, 1, 3.2, and 10 mg./l.; for Cl<sub>3</sub>POP, 0.1, 0.32, 1, and 3.2 mg./l.

sufficiently broad to demonstrate the most effective range for inducing normal roots. This in itself indicates that previous methods for testing and evaluating rooting responses were satisfactory for determining the effective concentration range of these substances. However, in view of the large number of variables in these and in previous tests, it is not always

possible to rank correctly a large number of compounds. For example, it was evident that the ranking of the compounds on a basis which included root counts for supra-optimal concentrations, as in the 1943 tests, would be different from that based on the effective range for inducing normal roots. Particularly in the cases where the results of duplicated tests are greatly different, additional tests are necessary. This was true for  $\text{Cl}_3\text{POA}$  in the 1943 tests. In the first of the duplicated tests the average number of roots for three concentrations was 32 and for the second test 4. The average, 18, of these two is not representative of the high activity of  $\text{Cl}_3\text{POA}$  shown in later tests including the present 1944 tests, in which each treatment was replicated four times.

### *Results Statistically Analyzed*

Root counts for series A, B, and C appear in Table III. The analysis of variance for these data appears in Table III. The seven acids in each series were significantly different ( $>P.01$ ) in their capacity to induce roots in privet cuttings. Relative differences in activity within each group of seven acids are shown in Table V. The separation into groups of high, medium, and low activity was made on the basis of the L.S.D. value at the 5 per cent level required for totals of 12 root counts (line totals in Table III). These results (Table V) show that only 2 of the 12 phenoxy acids ( $\text{Cl}_3\text{POA}$  and  $\text{Cl}_3\text{POP}$ ) were of equivalent activity to NA. Thus the trichlorophenoxy acids were of highest activity, the dichlorophenoxy acids of intermediate activity, and the monochlorophenoxy acids together with  $(\text{CH}_3)_2\text{POP}$  were of lowest activity. In general, these differences are reflected in the curves in Figures 1, 2, and 3.

The concentration effect was highly significant for the acids in each series of tests (Table III). This was to be expected since the three concentrations selected from a total of four were from the optimal part of the concentration curve. However, significant  $F$  values for the interaction acid  $\times$  concentration indicated that the slopes of the concentration curves (Figs. 1, 2, and 3) were different for the monochlorophenoxy acids as compared with the di- and trichlorophenoxy acids and NA. For example, an  $F$  value of 2.09 for acids of two and three substituents (series B) was considerably less than the  $F$  values for the same interactions which included acids of one substituent (8.75 and 8.52 respectively for series A and C). These results are in agreement with those of previous tests (2).

Four of the acids used in series A were also used in series B. A comparison of the root counts for the replicated series of tests yielded a non-significant  $F$  value of 2.10. This indicated that the performance of these four acids which had two or three chlorine substituents was not significantly greater than the error of the replicated series. However, the  $F$  value of 2.87 for the interaction acid  $\times$  concentration was more nearly com-

parable to the  $F$  value in series B (2.09) than in series A (8.75). This indicates that the slopes of the concentration curves are more nearly alike for acids with two or three substituents than for acids of one substituent.

Differences due to the type of cutting were significantly different in series B and C but not in the case of series A (Tables III and IV). This is to be explained on the basis that the most comparable material was removed from the plants when the first collection was made for series A. Segregation of cuttings into four types was done for the purpose of treatment replication and not in order to demonstrate possible differences in type.

Since in previous tests the comparative activity of root-inducing

TABLE IV  
COMPARISON OF RESULTS OF THE ANALYSIS OF VARIANCE AND THE METHOD OF RANKS

	D.F.	Series A		Series B		Series C	
		$F$	$\chi^2$	$F$	$\chi^2$	$F$	$\chi^2$
Acid	6	19.32**	40.72**	12.43**	47.13**	25.68**	33.35**
Concentration	2	77.32**	14.00**	103.90**	14.00**	84.65**	14.00**
Type	3	1.50	6.25	12.83**	17.23**	9.95**	12.77**

\*\* Highly significant.

substances has been evaluated on the basis of ranked root counts, it seemed of interest to analyze the present results by a simplified statistical method such as the method of ranks devised by Friedman (1). An example of the procedures used for obtaining  $\chi^2$  values is given in Table II for data in series A. When the seven acids were classified by 12 root counts ( $n=12$ ,  $p=7$ ) a significant  $\chi^2$  value of 40.72 showed that these acids were unlike for inducing roots on privet cuttings. Additional comparisons within the group of seven acids were then made in order to determine which acids were of similar activity and which ones were significantly different. When the five acids which yielded column totals ranging from 39 to 66.5 were re-ranked ( $n=12$ ,  $p=5$ ), a non-significant  $\chi^2$  value of 9.03 was obtained. These five acids were placed in the medium active group for series A in Table V. The next procedure was to re-rank NA together with the five acids just mentioned ( $n=12$ ,  $p=6$ ). The resulting  $\chi^2$  value of 29.11 indicated that NA was significantly better ( $>P.01$ ) than the five acids of medium activity. Consequently NA was placed in the highly active group (Table V). In a similar manner  $(CH_3)_2POP$  was found to be of significantly lower activity ( $>P.01$ ) than the five acids in the medium active groups. Thus  $(CH_3)_2POP$  was placed in the group of low activity (Table V). The same procedures were used to rank the acids in series B and C for which the results are given in Table V. The group ranking of acids is essentially the same for both methods of analysis (Table V).

TABLE V

GROUP RANKING OF ACIDS BY THE ANALYSIS OF VARIANCE AND THE METHOD OF RANKS

Level of activity*	Series A		Series B		Series A+B		Series C	
	Rank		Rank		Rank		Rank	
	Var.	$\chi_r^2$	Var.	$\chi_r^2$	Var.	$\chi_r^2$	Var.	$\chi_r^2$
High	NA Cl <sub>2</sub> POP	NA	Cl <sub>2</sub> POA Cl <sub>2</sub> POP NA	Cl <sub>2</sub> POA Cl <sub>2</sub> POP NA	NA Cl <sub>2</sub> POP	NA Cl <sub>2</sub> POP	NA	NA
Medium	Cl <sub>2</sub> POP 3-CIPOP 4-CIPOP	Cl <sub>2</sub> POP** Cl <sub>2</sub> POP 3-CIPOP 4-CIPOP 2-CIPOP	Cl <sub>2</sub> POP Cl <sub>2</sub> POB Cl <sub>2</sub> POB	Cl <sub>2</sub> POP Cl <sub>2</sub> POB Cl <sub>2</sub> POB	Cl <sub>2</sub> POP	Cl <sub>2</sub> POP	4-CIPOP 3-CIPOP 2-CIPOP 4-CIPOP 3-CIPOP	4-CIPOP 3-CIPOP 2-CIPOP 4-CIPOP
Low	2-CIPOP (CH <sub>3</sub> ) <sub>2</sub> POP	(CH <sub>3</sub> ) <sub>2</sub> POP	(CH <sub>3</sub> ) <sub>2</sub> POP	(CH <sub>3</sub> ) <sub>2</sub> POP	(CH <sub>3</sub> ) <sub>2</sub> POP	(CH <sub>3</sub> ) <sub>2</sub> POP	2-CIPOP	3-CIPOP 2-CIPOP

\* Significant difference at 5 per cent level in all series between high and medium active groups, but all acids in medium group not significantly different from acids in low group.

\*\* Note that the results for series A+B place this acid in the highly active group.

By reversing the procedure just described, the seven acids formerly used as variates were used as factors for classifying concentration and type of cutting. For the concentration effect based on the average for the four types ( $n=7$ ,  $p=3$ ) highly significant  $\chi_r^2$  values were obtained in series A, B, and C (Table IV, line 2). Additional comparisons (not shown) of the concentration effect for each of the four types of cuttings ( $n=7$ ,  $p=3$ ) gave significant  $\chi_r^2$  values in 11 of 12 cases. The results for differences in

TABLE VI

CORRELATION OF THE RELATIVE ORDER OF ACTIVITY OF THE ACIDS TESTED IN 1943 AND 1944

Code name of acid	1944 tests		1943 tests	
	Total number roots*	Rank	Rank	Average number roots
Cl <sub>2</sub> POA	343	1	5	18
NA	304	2	2	26
Cl <sub>2</sub> POP	273	3	1	27
Cl <sub>2</sub> POB	178	4	6.5	14
Cl <sub>2</sub> POP	166	5	3	24
Cl <sub>2</sub> POB	148	6	8	12
3-CIPOP	109	7	9	10
4-CIPOP	106	8	11	8
2-CIPOP	105	9	6.5	14
4-CIPOP	88	10	12	7
3-CIPOP	72	11	4	21
(CH <sub>3</sub> ) <sub>2</sub> POP	69	12	10	9
2-CIPOP	61	13	13	5

$$r_r = 1 - \frac{6\sum D^2}{n(n^2-1)} = 1 - \frac{663}{2184} = .6964 \text{ (equivalent to } r = .713) **$$

\* Averages used in cases of duplication.

\*\* (5, p. 472).

type of cutting ( $n=7$ ,  $p=4$ ) appear in line 3 of Table IV. These results likewise agree with those obtained by the analysis of variance (Table IV).

The relative order of activity of the acids tested in 1944 was approximately the same as in the 1943 tests (Table VI). Since there were more replicated treatments in the 1944 tests, it is believed that the relative order of activity is more nearly correct in these later tests in which  $\text{Cl}_3\text{POA}$  and  $\text{Cl}_3\text{POP}$  proved more effective than  $\text{Cl}_2\text{POP}$ .

#### DISCUSSION

Considering the many substances known to possess root-inducing properties, there appear to be relatively few which from all standpoints are as satisfactory as NA and IB. Only two of the phenoxy acids ( $\text{Cl}_3\text{POA}$  and  $\text{Cl}_3\text{POP}$ ) proved to be of equal effectiveness to NA for inducing normal roots in privet cuttings (Table V). The dichlorophenoxy acids were of intermediate effectiveness, and the monochlorophenoxy acids together with  $(\text{CH}_3)_2\text{POP}$  were of lowest effectiveness. Although the phenoxy acids having one or two chlorine substituents were highly active, the effective range in concentration for inducing normal roots was narrower, and the maximum number of roots was less than for  $\text{Cl}_3\text{POA}$ ,  $\text{Cl}_3\text{POP}$ , and NA. On the basis of threshold concentrations, phenoxy acids having two or three chlorine substituents were from five to ten times more effective than NA (Figs. 1 and 2). This difference is in agreement with that reported previously for privet (2, p. 501), but this magnitude of difference does not hold for certain other species of plants (2).

The loss of information attending the use of ranking methods was not sufficiently large in the present tests to result in a radically different order of group ranking than was obtained by the analysis of variance (Table V). Considering this fact together with the simplicity and time-saving element of the ranking method, there appears to be justification in some cases for using the method of ranks in preference to the analysis of variance for determining differences between several root-inducing acids. The phenoxy acids having one or two chlorine substitutes received a lower rating mainly on the basis of adverse effects associated with the induction of relatively large numbers of roots. Thus certain responses which are difficult to measure quantitatively, such as swelling and proliferation of stems and fasciation of roots, are important in determining the effective concentration range of a given acid. The sum total of all responses on the cutting might be used as a basis for ranking which makes unnecessary the use of quantitative measurements, although of course the latter may also be used.

Even though the number of roots may not constitute an entirely satisfactory criterion for measuring differences in root-inducing activity, the results are reproducible in the same and in different tests. The relative

order of activity for the 13 acids tested in 1944 was approximately the same as in 1943 (Table VI). Any better correlation ( $r_r = .696 > P.01$ ) could scarcely be expected in view of the limited replication in the 1943 tests and also because of the rather large experimental error involved in this type of test.

#### SUMMARY

A complete list of the 63 phenoxy compounds tested in 1943 is given together with the average number of roots induced on cuttings of privet (*Ligustrum ovalifolium*). The results of these tests were described only briefly in a previous report.

Eighteen of the phenoxy acids used in 1943 were tested again in 1944 using a greater number of replicated treatments. The relative order of activity was approximately the same in both years' tests ( $r_r = .696 > P.01$ ). The relation between structure of acid and root-inducing activity was also essentially the same for both years' tests.

The group ranking of 12 of the active acids was essentially the same by Friedman's method of ranks and the analysis of variance. Only two of the acids [2,4,5-trichlorophenoxyacetic and  $\alpha$ -(2,4,5-trichlorophenoxy)-propionic acids] were of equivalent activity to NA. The dichlorophenoxy and most of the monochlorophenoxy acids were of intermediate activity.  $\alpha$ -(2,5-Dimethylphenoxy)-propionic and  $\alpha$ -(2-chlorophenoxy)-propionic acids were of lowest activity.

The loss of information attending the use of the method of ranks appears not to be a serious one in this type of test and is compensated for to a considerable extent by the simplicity and time-saving element of the method.

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# A NOTE ON THE PREPARATION OF 2-CHLORO-3,5-DIIODO-BENZOIC ACID AND 2-CHLORO-3,5-DIBROMOBENZOIC ACID AND THEIR EFFECTS ON TOMATO PLANTS

MARTIN E. SYNERHOLM AND P. W. ZIMMERMAN

One of the authors has been studying the physiological responses in plants to substituted benzoic acids. In the course of his work it was observed (1) that the product obtained in an attempted preparation of 2-chloro-3,5-diiodobenzoic acid possessed marked formative influences when applied either to the soil as an aqueous solution or as a solution in lanolin to local parts of the tomato (*Lycopersicon esculentum* Mill.) plant.

The attempted preparation consisted of a diazotization of 3,5-diiodoanthranilic acid in hydrochloric acid followed by replacement of the diazo group by chlorine. It was later shown that the product possessed the melting point and analysis for the unchanged diiodoanthranilic acid. Any physiological activity was due to traces of another compound, for the starting material was shown to be inactive. This led to a renewed effort to obtain the desired chloro derivative. After several unsuccessful attempts at diazotizing the diiodoanthranilic acid using hydrochloric acid as the diazotizing medium, it was found possible to carry out this step successfully using a mixture of glacial acetic acid and concentrated sulphuric acid. The replacement by chlorine followed smoothly according to the standard procedure. The product, 2-chloro-3,5-diiodobenzoic acid, has heretofore not been described in the literature.

The previously unreported 2-chloro-3,5-dibromobenzoic acid has now been prepared in an analogous manner.

*2-Chloro-3,5-diiodobenzoic acid.* A mixture of 20 g. (0.052 mol.) of 3,5-diiodoanthranilic acid in 50 ml. of glacial acetic acid and 50 ml. of concentrated sulphuric acid was cooled to  $-5^{\circ}$  C. in a freezing mixture. Three and six-tenths grams of sodium nitrite were added gradually while the mixture was stirred. After stirring for one hour in the cold, the mixture was poured with stirring onto 100 g. of ice. The solution was filtered to remove tar, and 100 ml. of concentrated hydrochloric acid and 50 g. of ice were added.

Cuprous chloride was prepared in the following way: Eighteen grams of copper sulphate pentahydrate and 5 g. of sodium chloride were dissolved in 60 ml. of hot water. To this solution was added with stirring a hot solution prepared by dissolving 3.8 g. of sodium bisulfite and 2.6 g. of sodium hydroxide in 30 ml. of hot water. The precipitated cuprous



chloride was washed several times by decantation, then taken up in 30 ml. of approximately 25 per cent hydrochloric acid, and cooled to 0°. The cuprous chloride solution so prepared was added to the diazotized anthranilic acid derivative and the mixture warmed slowly on the steam bath to 45°. The mixture was filtered, dried, and recrystallized from benzene. The yield was 13 g. (70 per cent) of material melting at 207 to 208° (uncorr.).

Neutral equivalent calculated for  $C_7H_5O_2ClI_2$ : 408.4. Found: 405.

*2-Chloro-3,5-dibromobenzoic acid.* This acid was prepared in an analogous manner from 10 g. of methyl 3,5-dibromoanthranilate in 40 ml. of glacial acetic acid and 20 ml. of concentrated sulphuric acid. Two and two-tenths grams of sodium nitrite, 11 g. of copper sulphate, 3 g. of sodium chloride, 2.3 g. sodium bisulfite, and 1.6 g. sodium hydroxide were used in the diazotization and replacement steps. The crude product was warmed for a half hour with 95 per cent alcoholic sodium hydroxide to effect complete saponification of the ester. The product after dilution with water, acidification and recrystallization from benzene, weighed 8.5 g. (84 per cent), melting point 180 to 181° (uncorr.).

Neutral equivalent calculated for  $C_7H_3O_2ClBr_2$ : 314.4. Found: 314.

Substituted benzoic acids differ physiologically from many other growth substances in that they cause little or no cell enlargement or induction of adventitious roots. They resemble the substituted aryloxy alkyl carboxylic acids in their capacity to cause formative effects (1, 2, 3) in low concentrations. Likewise, inhibition of growth and herbicidal activity are manifested at moderate and high concentrations of these chemicals. The positions of the substituents in the benzoic acid series are important in lending activity to the compound. For example, 2,5-dichlorobenzoic acid has a pronounced formative influence on plants, while 2,4-dichlorobenzoic acid is only slightly active. The formative effects of four substituted benzoic acids are illustrated in Figure 1. In the early stages of growth, the effects of the different acids are similar.

Several methods have been used in applying the chemicals to the plant, the simplest being the application of an aqueous solution of the chemical to the soil. It has usually been applied in amounts ranging from 1 to 20 mg. in 50 ml. of water for a four-inch pot of soil. One application shows activity lasting from one to six months depending upon the concentration and effectiveness of the compound used.

The compounds may be tested effectively by application of their lanolin solutions to one side of the stem and an adjacent leaf or as aqueous sprays to the growing tip and leaves. Still another method involves vaporization of the chemical by heat in a confined space such as within a closed bell jar or Wardian case.

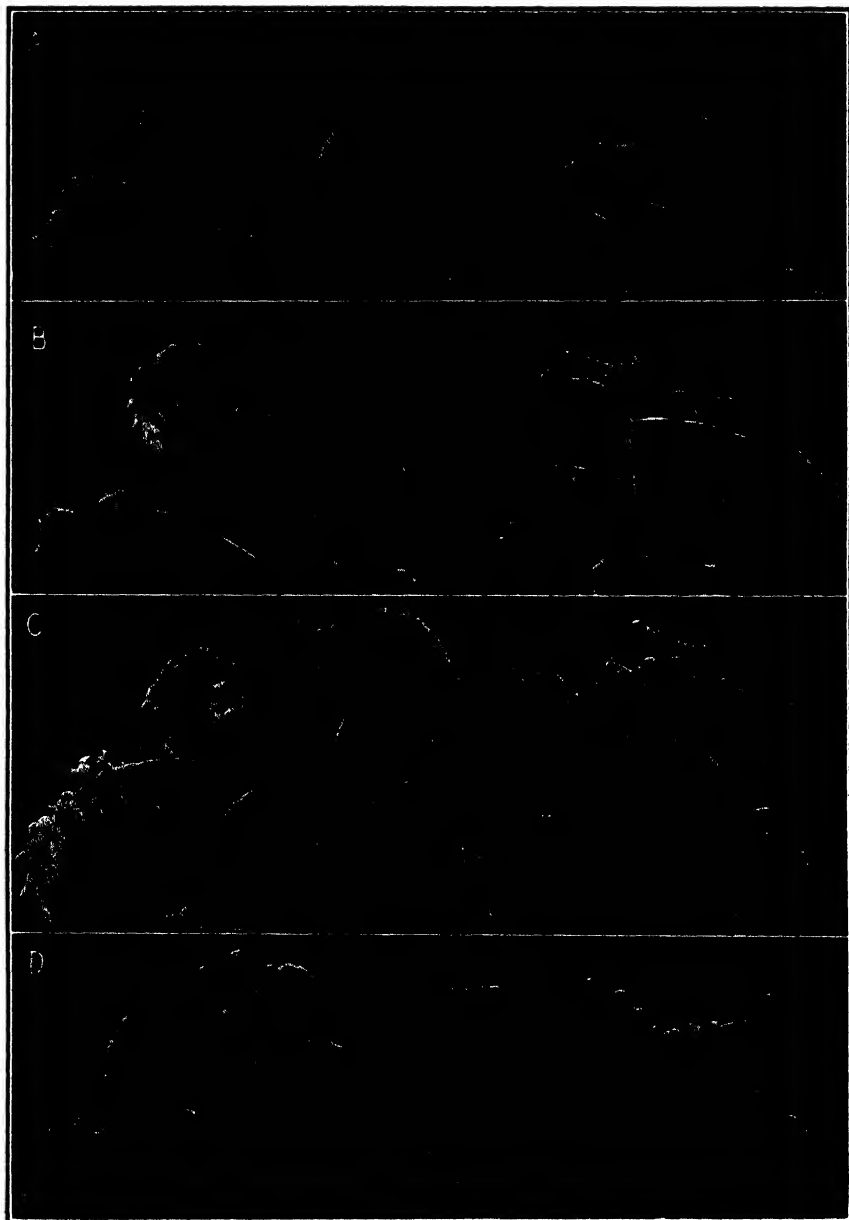


FIGURE 1. Tomato plants illustrating the formative influence of four halogen-substituted benzoic acids; photograph taken 17 days after 10 mg. of the acid in 50 ml. of water were applied to the soil of a four-inch pot in which the plant was growing. Plants were approximately four inches tall when treated. The acids used were (A) 2,3,5-triiodobenzoic acid; (B) 2-chloro-3,5-diiodobenzoic acid; (C) 2-chloro-3,5-dibromobenzoic acid; and (D) 2,5-dichlorobenzoic acid.

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## FAVORABLE CONDITIONS FOR THE TREATMENT OF DORMANT GLADIOLUS CORMELS TO INCREASE GERMINATION

F. E. DENNY

Previous reports (1, 2, 3) had shown that a favorable temperature for over-winter storage of cormels previous to treatment before planting in the spring was about 5° or 10° C. (41° or 50° F.), and that a suitable amount of the chemical, ethylene chlorohydrin ( $\text{CH}_2\text{ClCH}_2\text{OH}$ ), for use in the treating of cormels by exposure to the vapors of this chemical, was approximately 1 cc. of the 40 per cent solution of ethylene chlorohydrin for each 80 to 100 grams of cormels (equivalent to about seven drops of the chemical per ounce of cormels, or about one and one-fourth teaspoonfuls per pound, or about one pint to 100 pounds).

Subsequent experiments have confirmed the suitability of these conditions for pre-storage and treatment, and have indicated that the concentration of the chemical need not be very exact but can be varied from about 1 cc. of the 40 per cent solution to each 37.5 grams of cormels to about 1 cc. to each 125 g. without getting beyond the effective range in either direction. This indicates that the mid-point of the effective range is at a somewhat stronger concentration than that given for the 100-pound quantity in the preceding paragraph, and that the directions in this case should read one pint to each 80 pounds of cormels.

After the experiments were well along the question came up whether at the end of the treatment period it was necessary to plant the treated cormels at once or whether, in case the weather was unfavorable for planting, the treated cormels could be held for a few days and then be planted without serious loss of the effectiveness of the treatment. The preliminary test, using a delay period of four days, indicated that not only was there not a loss but there was, in reality, a gain due to this delay after treating and before planting. A test in the following year, in which the delay periods used were one week and two weeks, again indicated a gain due to delayed planting and, in fact, suggested that the two weeks' delay period was perhaps more favorable than that of one week.

The present experiment was undertaken to determine more definitely whether there was a gain in yield by delaying the planting after treatment, and, if so, whether the conditions of storage of the treated cormels during the period of delay were of importance in the effect of the delay. For this purpose the concentration of the chemical used in the treatment and the temperature of storage of the cormels during the period of delay were both varied over a considerable range, and the possible effect of the moisture conditions during the delay period was tested by employing both dry and moist storage.

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The result has been to show that a delay period of six days after treatment before planting was favorable, that a delay of 12 days was more favorable than one of six, but that a delay of 18 days did not still further increase the yield, although in this case the yield of the 18-day delay lots was greater than that of the lots planted at once after treatment.

#### MATERIAL AND METHODS

The *Gladiolus* cormels, which were of the variety Salmon Star, were of the crop of 1943 and were stored over-winter in paper bags at a temperature of 5° C. (41° F.). Late in April 1944 they were sorted into lots weighing 12.5 grams each, averaging 105 cormels per lot, and tied in individual cheesecloth bags. To provide for all of the treatments now to be described, 216 of these bags of cormels were required.

The main object of the present test was to determine whether after the completion of a treatment with the vapor of ethylene chlorohydrin the cormels should be planted at once, or whether a better yield would be obtained by delaying the planting after treatment. Three delay-periods were used, 6 days, 12 days, and 18 days, and for comparison with them were lots treated in the same way except that they were planted at once after treatment.

Three different concentrations of chemical were used for the four-day period of treatment: 1 cc. of 40 per cent ethylene chlorohydrin to each 50 grams of cormels, 1 cc. per 75 grams, and 1 cc. per 100 grams. The bags of cormels were put into quart-size glass fruit jars of the type with wide openings. When the jar was nearly full a piece of a paper towel was laid on top of the bags, and on this was laid a piece of cheesecloth containing the proper amount of chemical as determined by the weight of cormels in the jar. The 40 per cent solution was prepared from the anhydrous chemical by adding 1.75 volumes of water to one volume of the anhydrous chemical and mixing thoroughly.

The temperature during the four-day duration of the treatment was 22.5° C. (72.5° F.).

During each of the periods of delay after treatment before planting, three different temperatures for storing the treated cormels were used: 20° C. (68° F.), 25° C. (77° F.), and 30° C. (86° F.).

A still further subdivision was made to determine the effect of the storage condition at each temperature, one series of lots being stored in air in a shallow layer in a wooden flat, and the other series being removed from the bags and mixed thoroughly with moist sand and stored in wide-mouthed stoppered bottles.

The number of lots of bulblets required for a single replication was, therefore,  $4 \times 3 \times 3 \times 2 = 72$ , and since the entire series of treatments was replicated three times in the field planting, the total number of lots of cormels was 216.

The treatments were started at intervals such that all lots would finish their periods for treatment plus delay on the same day, so that all lots could be planted simultaneously. Thus, by starting one set of lots on April 23, another on April 29, another on May 5, and another finally on May 11, all lots were ready for planting on May 15.

For the planting plan in the field the split-plot method (4, p. 209) was used. The portion of the field for each replication was divided into three equal areas to receive the lots receiving the three different concentrations of chemical, or nine such areas for the three replications. Each of these nine areas was divided into three equal areas to receive the lots that had been stored at the three different temperatures after the treatment had been applied, there being thus 27 such areas. Each of these in turn was divided into two equal areas to receive the lots stored under the two different conditions, i.e., in air and in moist sand, there being 54 such areas. Finally each of these last named areas was divided into four equal areas to receive the lots that were subjected to the four delay periods after the treatments had been applied, i.e., 0, 6, 12, and 18 days.

Each lot occupied a three-foot portion of a row, the bottom of the furrow being broad so as to allow the bulblets to be scattered in a band about three inches wide in the row.

This planting plan allowed the effect of the period of delay after treating before planting to be determined more accurately than any of the other factors, since these lots were always adjacent to each other in the field, and so were less subject to differences on account of soil variation. The effect of other factors was consequently less accurately determined, and in the following order of decreasing accuracy: storage condition (dry or moist), temperature, and finally concentration of chemical used in the treatments.

No controls, or non-treated bulblets, were included in this planting plan since it had been found in various tests in previous years that cormels of this variety after over-winter storage even at the favorable temperature of 5° C. (41° F.) would germinate poorly. Nevertheless, ten lots, of the same stock as those used in the treatments, were planted in rows adjacent to the treated lots in the field in order to verify the fact that the cormels used in the test were dormant. As expected, the germination of these check lots was poor and the average yield of young corms per lot of 105 cormels was only 10.2 g. while in the treated lots the average yield of all lots under all conditions of treatment was 128.8 g.

## RESULTS

The young corms were harvested, the tops, roots, and young cormels were rejected, and the weight in grams of the crop was recorded for each



TABLE I

YIELD IN GRAMS OF CORNS FROM EACH LOT (12.5 G., OR 105 CORNELS) OF GLADIOLUS CORNELS TREATED WITH ETHYLENE CHLOROHYDRIN, STORED UNDER DIFFERENT CONDITIONS AFTER TREATMENT, REMOVED FROM STORAGE AFTER DIFFERENT INTERVALS, AND PLANTED IN THE FIELD

Temp. during storage	Dura- tion of storage, days	Stored dry, in air									Stored in moist sand									Totals of lines
		Concentration of chemical during treatment, grams of cornels per cc. of chemical									Concentration of chemical during treatment, grams of cornels per cc. of chemical									
		50			75			100			50			75			100			
		I	II	III*	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	
20° C. 68° F.	0	99	150	82	116	129	110	135	131	110	119	108	101	133	135	106	131	109	113	2117
	6	142	127	106	139	150	126	150	123	115	118	111	104	102	110	110	121	139	87	2234
	12	121	202	82	133	114	126	160	146	106	146	133	105	109	100	143	163	142	114	2345
	18	150	174	87	140	118	109	120	160	112	115	157	109	191	92	93	171	172	99	2378
25° C. 77° F.	0	76	125	114	131	146	105	130	134	84	112	119	112	139	122	98	170	128	95	2140
	6	128	152	112	172	136	112	148	155	90	108	114	87	100	134	144	158	124	122	2286
	12	153	146	100	186	99	120	117	158	79	159	169	114	109	157	106	165	167	117	2421
	18	122	160	134	160	118	130	134	129	80	124	172	123	200	139	104	144	106	143	2422
30° C. 86° F.	0	91	131	116	124	93	74	116	108	114	94	112	119	117	103	75	136	153	135	2001
	6	115	229	123	147	126	78	168	133	95	111	142	139	173	148	78	134	134	150	2398
	12	163	146	132	177	133	87	141	143	172	166	169	145	155	163	88	164	143	141	2628
	18	153	200	127	142	136	72	122	162	86	114	145	130	204	101	88	169	140	160	2451

\* The numerals I, II, and III indicate the three replications in the field plantings.

Note: The yields of each of the 10 plots of untreated (check) cornels planted in rows adjacent to these treated lots were as follows (in grams): 18, 7, 8, 4, 10, 15, 12, 11. Average = 10.2 grams.

lot. The results are shown in Table I. The analysis of variance of the data in Table I is shown in Table II, account being taken of the fact that only the lots with planting delayed after treatment were subjected to differences in storage temperature and media.

On account of the arrangement of the planting on the split-plot basis, there is more than one error term, four in this case, one for each of the main factors and their interactions. The error terms are each obtained from the combined interactions involving replications. Presumably, in

TABLE II  
ANALYSIS OF VARIANCE OF DATA IN TABLE I

Source	D.F.	Variance
Replications	2	20026
Concentrations of chemical	2	700
Error "a"	4	3747
Temperatures during storage	2	1299
Temp. $\times$ concn.	4	798
Error "b"	12	953
Medium during storage	1	4
Med. $\times$ concn.	2	1209
Med. $\times$ temp.	2	250
Med. $\times$ temp. $\times$ concn.	4	258
Error "c"	18	657
Delay in planting	3	4738**
Delay $\times$ concn.	4	445
Delay $\times$ temp.	4	179
Delay $\times$ med.	2	895
Delay $\times$ temp. $\times$ concn.	8	322
Delay $\times$ med. $\times$ concn.	4	419
Delay $\times$ med. $\times$ temp.	4	99
Delay $\times$ med. $\times$ temp. $\times$ concn.	8	308
Within concn., without delay	51	381
Error "d"	74	497

\*\* Prob.  $>.01$ .

the case of error "d," the three-factor and four-factor interactions involving "delay" could have been combined with the replication interactions to form a different error term. This would have given an error variance of 448 with 98 degrees of freedom. However, the results of the tests of significance would not have been changed by the use of this error term.

The only factor showing a significant effect with odds of 19 to 1 or more is that due to the delay in planting after treatment, the  $F$  value (4, p. 254) being 9.53, corresponding to odds well beyond the 99 to 1 point. The totals for the 54 lots planted at once (0 days) and those subjected to a delay of 6, 12, and 18 days, also each consisting of 54 lots, were as follows in the order given: 6258 g., 6918 g., 7394 g., and 7251 g. In the comparison

o vs. 6 days, the  $F$  value (5, p. 339) is  $(6918 - 6258)^2 \div (54 \times 2 \times 497) = 7.97$ , corresponding to odds well over 99 to 1 that on the whole a delay of 6 days after treatment before planting increased the yield as compared with planting at once after treatment. In the comparison 12 days' delay vs. 6 days' delay, the  $F$  value is  $(7394 - 6918) \div (54 \times 2 \times 497) = 4.14$ , which indicates odds of slightly more than 19 to 1 that a delay of 12 days was more favorable than a delay of 6 days after treatment before planting. A similar comparison of the values for 18 days vs. 12 days fails to show a difference but in the comparison o vs. 18 days, the  $F$  value is 18.0, showing high odds that a delay of 18 days in planting gave a better yield than that obtained by planting at once after treatment.

The totals for the effect of the storage medium after treating and before planting were as follows (81 lots in each total): stored dry in air, 10,769 g.; stored in moist sand, 10,794 g.

The failure of the different temperatures during the storage after treatment to show a large effect was unexpected. The totals for the 54 lots at each temperature were as follows for 20° C. (68° F.), 25° C. (77° F.), and 30° C. (86° F.), in the order given: 6957 g., 7129 g., and 7477 g. There is an increasing series here, but the analysis of variance shows that on the whole the differences are not significant, at least with odds of 19 to 1.

The totals for the three concentrations of chemical, 1 cc. to 50 g., 1 cc. to 75 g., and 1 cc. to 100 g. (72 lots at each concentration), were as follows, in the order given: 9289 g., 9042 g., and 9490 g. These values are not in a series, the intermediate concentration being lower than that of either the weakest or strongest, but the analysis of variance does not indicate a significant difference.

The conclusion is that a delay of at least 6 days and as many as 18 days after treating and before planting gave a better yield than was obtained when the cormels were planted at once after treatment.

#### SUMMARY

*Gladiolus* cormels of the crop of 1943 were stored over-winter at 5° C. (41° F.) and were treated late in April 1944 with the vapor of ethylene chlorohydrin, using three different concentrations of chemical as follows: 1 cc. of 40 per cent ethylene per 50 g. of cormels, 1 cc. per 75 g., and 1 cc. per 100 g. After the treatments were completed, one series of treated cormels was planted at once in the field, another series was stored for 6 days after treatment before being planted, while other lots were subjected to a delay in planting of 12 days and 18 days. The treatment of the various lots was initiated at the proper intervals before planting so that the periods of treatment plus storage after treatment terminated simultaneously, permitting all lots to be planted on the same day.

During the intervals of storage after treatment, three temperatures

of storage were tested: 20° C. (68° F.), 25° C. (77° F.), and 30° C. (86° F.), and at each of these temperatures there were two series, one in which the treated cormels were stored dry in air, and the other in which they were stored in moist sand.

The yield of lots subjected to a delay of 6 days after treatment was greater than that of lots planted at once after treatment, a delay of 12 days was better than one of 6, and a delay of 18 days, although not giving more favorable results than one of 12, resulted in a greater yield than was obtained with treated lots planted without delay.

These favorable results of a delay in planting after treatment were obtained under all conditions of treatment and subsequent storage tested in the experiment.

From these results, and those of previous experiments, the favorable conditions for obtaining good germination of gladiolus cormels are as follows: store the cormels over-winter at a temperature of 5° C. (41° F.) to 10° C. (50° F.). Start the chemical treatment 10 to 20 days before it is planned to plant. Treat the cormels in containers that can be closed, using 1 cc. of 40 per cent ethylene chlorohydrin for each 75 g. of cormels (seven drops per ounce, or one and one-fourth teaspoonfuls per pound, or one pint per 80 pounds), incorporating the chemical into cheesecloth of a size sufficient to avoid dripping, spreading the cloth loosely on a piece of paper toweling at the top of the container. Seal the container and let it stand for four days at room temperature (approximately 22° C., or approximately 72° F.), avoiding temperatures below 20° C. (68° F.) or above 30° C. (86° F.). Remove and store the treated cormels in air at room temperature for one to two weeks and then plant. If the ethylene chlorohydrin available is the anhydrous chemical, prepare the 40 per cent solution from this by adding one and three-fourths volumes of water to one volume of the anhydrous chemical and mix thoroughly. If large amounts of cormels are treated in closed rooms, avoid breathing strong concentrations of the vapor, and at the end of the treatment open up the closed space and ventilate it thoroughly before entering the room to remove the treated cormels.

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# PRELIMINARY OBSERVATIONS ON THE TRANSLOCATION OF SYNTHETIC GROWTH SUBSTANCES

MARIO G. FERRI<sup>1</sup>

## INTRODUCTION

It has been shown by Hitchcock and Zimmerman (14) that synthetic growth substances can be absorbed from the soil by plants and transported upward as are mineral salts. The addition of those substances to the soil proved to be the most effective method to induce the formation of adventitious roots on stems of tomato and tobacco plants. Indolepropionic acid even caused roots to appear over the petiole and along the midrib of leaves of tomato.

To detect the presence of the growth substances in tissues a certain distance from the place of application, Hitchcock and Zimmerman used several responses of aerial parts, such as bending, proliferation, root formation on stems, etc.

Since the upward movement of the applied growth substance was affected by conditions influencing the rate of transpiration (14, 15), it was suggested that the movement occurred in the transpiration stream. The fact that growth substances were able to move through dead tissues supported that idea. Furthermore, those substances could be absorbed by a slit portion of stem containing a sliver of wood, but not if the bark alone was immersed in the solution.

The present paper deals with the translocation of synthetic growth substances which have been applied to intact plants either as solutions poured into the soil or as lanolin preparations applied around the stem. It is shown that in both cases the upward movement was intense enough to induce rooting of leaf cuttings taken from the plant a certain time after treatment and planted in sand. Experiments made with hardwood cuttings gave some more direct evidence that the transport takes place mainly, if not exclusively, through the xylem.

The present results may have some application on the general problem of translocation of solutes. This question was open to discussion by Curtis (5) who, basing conclusions mainly on results obtained with ringing experiments, claimed that the upward as well as the downward movement of foods is made through the phloem and not through the xylem. He maintained this view in a series of papers and the idea of transport through the phloem, in either direction, was extended to mineral elements (6, 7, 8, 9). According to Curtis, then, the xylem is mainly concerned with the transportation of water.

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Dixon (10) and Dixon and Ball (11) on the other hand defended the view that both upward and downward transport of organic as well as of inorganic material takes place in the wood. Their conclusion was based mainly on calculations of rates of movement.

The opinion of several authors in relation to this problem lies between these two extremes. Thus, Maskell and Mason (18, 19, 20, 21) showed that the upward transport of inorganic nitrogen, phosphorus, potassium, and probably some other ash constituents, as well as of carbohydrates, is through the wood, while organic nitrogen and carbohydrates move downward through the phloem. Crafts (4) also admits that certain solutes, chiefly organic, are carried in both directions through the phloem, while others, mainly mineral salts, are carried through the xylem. Clements and Engard (2) criticized Curtis' technique and presented good evidence supporting the idea that the xylem is the main path of transport of salts.

Once more the problem was reopened by Gustafson and Darken (13) who, working with radioactive phosphate, showed upward movement through both wood and bark.

More recently Stout and Hoagland (22), using radioactive isotopes not only of phosphorus, but also of potassium and sodium, concluded in favor of the xylem as the channel concerned with the longitudinal movement of mineral elements. From the wood the radioactive elements can diffuse radially to the bark, but longitudinal movement through the bark was not observed.

It had already been emphasized by Hitchcock and Zimmerman (14) that the use of synthetic growth substances might give a clearer idea about the whole problem of translocation of materials in plants, since they can be detected in extremely small amounts and their use avoids the complications introduced by the techniques employed so far.

#### MATERIALS AND METHODS

The principal growth substances used in the present experiments were  $\beta$ -indolebutyric and  $\alpha$ -naphthaleneacetic acids. Indoleacetic and 2,4-dichlorophenoxyacetic acids, potassium indolebutyrate, and potassium naphthaleneacetate were also employed. Fresh solutions of these substances, at the desired concentrations, were prepared for each test. Lanolin preparations of naphthaleneacetic acid at the concentrations of 5, 10, and 20 mg. per g. were also used.

Leaf cuttings of tomato (*Lycopersicon esculentum* Mill. var. Bonny Best) and *Cleome spinosa* L., and hardwood cuttings of *Hibiscus syriacus* L. were used as test objects.

In the case of leaf cuttings, 50 cc. of solution were poured on the soil of potted plants (4-inch pots). After a certain period of time three (tomato) or five (*Cleome*) leaves were taken from the plant. The petioles were cut close

to the stem. The lowest leaflets were removed and those remaining were trimmed to reduce and equalize the surfaces. The cuttings were then planted in sand in a greenhouse bench.

Whenever lanolin preparations were used, the lanolin was applied around the stem with a glass rod for a distance of about 2.5 cm. The place of application and the time after which cuttings were made and planted, as well as the region (above or below the treated zone) where cuttings were taken, will be specified with the description of the experiments.

Control cuttings, from plants as comparable as possible to those treated, were planted at the same time, under the same conditions.

Root counts were made about eight days after cuttings had been in the rooting medium. In the case of lanolin treatment this period was in general longer, about twelve days.

Segments of stems of tomato plants were killed by heat. The potted plant was held in the horizontal position and turned over a microflame. The dead segment was about 3 cm. wide beginning at 5 cm. from the soil surface. In a short time the dead segment shrank and showed a brown color. By lack of turgidity it could not sustain the upper part of the plant which bent down. The solution of the growth substance was poured into the soil shortly after the segment was killed.

In some other tests stems of tomato plants bearing all leaves were cut near the soil surface and their bases were immersed in boiling water to a depth of 3 cm. for two minutes. The killed region was, however, much broader—about 8 cm.—due to the effect of the hot water vapor. The bases of these stems were then dipped into a solution of a growth substance to a depth of 2 cm. Cuttings taken from these plants were prepared and planted as indicated above.

In the case of *Hibiscus* cuttings, 50 cc. of solution were placed in 250 cc. beakers and the basal ends of the cuttings were immersed to a depth of 2 cm. Some of these cuttings were left with both wood and bark; some were deprived at the base of either the wood or the bark for a distance of about 5.5 cm. In every case a pure lanolin band 1 cm. wide was applied immediately above that region (in the cuttings with bark and wood at an equivalent distance from the base) to avoid a possible external rise of the chemical by capillarity. At the end of 24 or 48 hours, as will be specified, the cuttings were recut above the lanolin band (about 6 cm. from the base) and planted in sand in the greenhouse. Control cuttings remained in tap water during the period of treatment and were also recut before planting. As a previous test showed, there was no difference in rooting of controls with or without the band of lanolin; this was not applied to them in further experiments. The time after which root counts were made varied around 20 days, depending upon the type of cutting.

The size of cuttings at the time of treatment was in general 17.5 cm.



and at the time of planting, after they had been recut, was about 11 cm. Each lot was composed in general of three cuttings. Attempts were made to have lots as comparable as possible, although in each lot cuttings might vary in diameter, number of buds present, position in the whip from which they had come, etc. Any important alteration concerning the method of treatment or the type of cutting will be stated with the description of the experiments.

### RESULTS AND DISCUSSION

The preliminary experiments are a combination of experiments previously made by other authors who studied either the absorption and transport of growth substances applied to the soil of potted plants by reactions of aerial parts such as bending, stem proliferation, etc. (14), or the

TABLE I

RESULTS OBTAINED WITH TOMATO LEAF CUTTINGS AFTER APPLICATION OF INDOLEBUTYRIC ACID TO THE SOIL. LENGTH (MM.) OF PETIOLE FROM WHICH ROOTS EMERGED 9 DAYS AFTER PLANTING. CUTTINGS MADE 20 HOURS AFTER PLANTS HAD BEEN TREATED

Amount of chemical (mg. per pot)	Plants 11 inches tall*	Plants 7 inches tall**	Average
0	0	0	0
1.0	0	2.8	1.4
3.2	5.5	1.8	3.6
10.0	16.2	48.9	32.5
32.0	76.1	89.1	82.6

\* Averages of 3 plants (9 cuttings).

\*\* Averages of 2 plants (6 cuttings).

rooting caused by direct treatment of cuttings (16, 17). In the present study the rooting of cuttings not directly treated, but taken from treated plants, was observed.

Five lots of tomato plants, each including three plants about eleven inches tall and two, seven inches tall, were selected, care being taken in order to get lots as comparable as possible. Each treated plant in every lot received 50 cc. of solution of indolebutyric acid. The concentrations used were: 0 (tap water controls), 1.0, 3.2, 10.0, 32.0 mg. per 50 cc. of water. Twenty hours after treatment, the three uppermost adult leaves were severed from each plant, prepared and planted as indicated under the heading Materials and Methods. After nine days in the rooting medium the cuttings were removed and the length of the petiole from which roots emerged was measured. The results of this test are reported in Table I.

These results show first that there is an upward transport of the synthetic growth substance applied to the soil; second, that the amount of chemical transported is enough to influence rooting of leaf cuttings; and

third, that there is a direct relation between the concentration and the amount of chemical put into the soil and the response of the cuttings. The lowest concentration did not show a marked influence. With the highest concentration roots are shorter, much more numerous, and emerging from a longer distance from the base (Fig. 1 A).

Experiments comparable to the one just described were made to test other substances. The results may be summarized as follows:  $\alpha$ -naphthaleneacetic acid was more effective than indolebutyric acid at the lowest concentration (1 mg. per 50 cc.); at the two highest concentrations (10 and 32 mg. per 50 cc.), however, indolebutyric acid was more effective. This result parallels those obtained by Hitchcock and Zimmerman (17). They observed that with talc preparations directly applied to cuttings of *Thuja occidentalis* var. *globosa*, naphthaleneacetic acid was active at lower concentrations but indolebutyric acid had a broader range of effectiveness; and that in *Euonymus* the mixtures (indolebutyric+naphthaleneacetic, indolebutyric+indoleacetic, and indolebutyric+phenylacetic acids) were less effective in the range 44 to 80 mg. per liter but more effective in the range 16 to 44 mg. per liter than indolebutyric acid (17, p. 153).

Indoleacetic acid was less active than either indolebutyric or naphthaleneacetic acids; the minimum effective amount was, under the conditions of the present experiments, 10 mg. per pot. One hundred mg. of indoleacetic acid were about as effective as 32 mg. of indolebutyric acid. Although obtained with a different method of treatment, these results are in agreement with those obtained by Hitchcock and Zimmerman (15) with direct application which showed that indolebutyric and naphthaleneacetic acids were more active than indoleacetic acid.

2,4-Dichlorophenoxyacetic acid, on the other hand, showed a much narrower range of activity; 1 mg. per pot gave maximum results, being about as effective as 32 mg. of indolebutyric acid. Even this concentration proved to be excessive in several instances depending upon the size, age, and other conditions of the plant (Fig. 1 B). One-tenth mg. of this compound in the soil (4-inch pot) was enough to induce rooting above the base on tomato leaf cuttings taken about 24 hours after treatment.

Another plant, *Cleome spinosa*, has also been tested. Table II shows the results of two experiments made in duplicate, one for indolebutyric, the other for naphthaleneacetic acid. The *Cleome* plants treated with indolebutyric acid were about 6 inches tall, whereas those treated with naphthaleneacetic acid were about 8 inches tall. In every case each plant received the amount of chemical to be tested, in 50 cc. of water. Cuttings were made 68 hours after treatment with indolebutyric acid and only 24 hours after treatment with naphthaleneacetic acid. The results of one are not then to be quantitatively compared with the results of the other. Only the qualitative differences can be stated. Root counts were made 17 days

after treatment with indolebutyric acid and 14 days after treatment with naphthaleneacetic acid.

With indolebutyric acid there is first an increase in number of roots proportional to the concentration, up to 10 mg. per 50 cc.; above this there is a sudden decrease. With naphthaleneacetic acid there is a continuous increase up to the highest concentration used, but at this concentration, while the number of roots is much greater, their length is smaller than with 10 mg. per 50 cc.; there is inhibition of root formation at the base of the petiole; roots emerge from all the length of the petioles and in one case

TABLE II

NUMBER OF ROOTS INDUCED ON CLEOME LEAF CUTTINGS AFTER APPLICATION OF SYNTHETIC GROWTH SUBSTANCES TO THE SOIL

Amount of chemical (mg. per pot)	Indolebutyric acid			Naphthaleneacetic acid		
	Plant 1*	Plant 2*	Average**	Plant 1*	Plant 2*	Average**
0	0.2	2.2	1.2	1.2	0.6	0.9
1.0	2.6	1.6	2.1	1.6	2.0	1.8
3.2	4.0	5.6	4.8	2.4	4.4	3.4
10.0	5.8	6.6	6.2	5.2	6.4	5.8
32.0	1.4	0	0.7	***	***	***

\* Averages of 5 cuttings.

\*\* Averages of 10 cuttings.

\*\*\* Number of roots so great that counting was not practical.

roots emerged from the midrib of a leaflet; with all concentrations below that, rooting was strictly basal as was the general case for indolebutyric acid (Fig. 1 C and D).

These results obtained with *Cleome* confirm those observed with tomato in showing that a plant can absorb from a solution poured into the soil, and transport upward, synthetic growth substances in an amount sufficient to influence the rooting of leaves taken from the treated plant. In this case there is good indication that the movement occurs in the transpiration

FIGURE 1. A, B, and E, tomato cuttings; C and D, *Cleome* cuttings. A, B, C, and D, cuttings made from plants treated with a solution of growth substance; in each case the amount of chemical indicated was poured on the soil in 50 cc. of water. E. Cuttings made from plants treated with lanolin preparations. Controls always at left. A. Cuttings made 20 hours after treatment with indolebutyric acid: 0, 1.0, 3.2, 10.0, 32.0 mg., 9 days after planting. B. Cuttings made 24 hours after treatment with 2,4-dichlorophenoxyacetic acid: 0, 0.1, 0.3, 1.0 mg., 9 days after planting. C. Cuttings made 68 hours after treatment with indolebutyric acid: 0, 1.0, 3.2, 10.0, 32.0 mg., 17 days after planting. D. Cuttings made 24 hours after treatment with naphthaleneacetic acid: 0, 1.0, 3.2, 10.0, 32.0 mg., 14 days after planting. E. Cuttings made 96 hours after treatment with naphthaleneacetic acid: 0, 5, 10, 20 mg. per g. of lanolin, 12 days after planting.

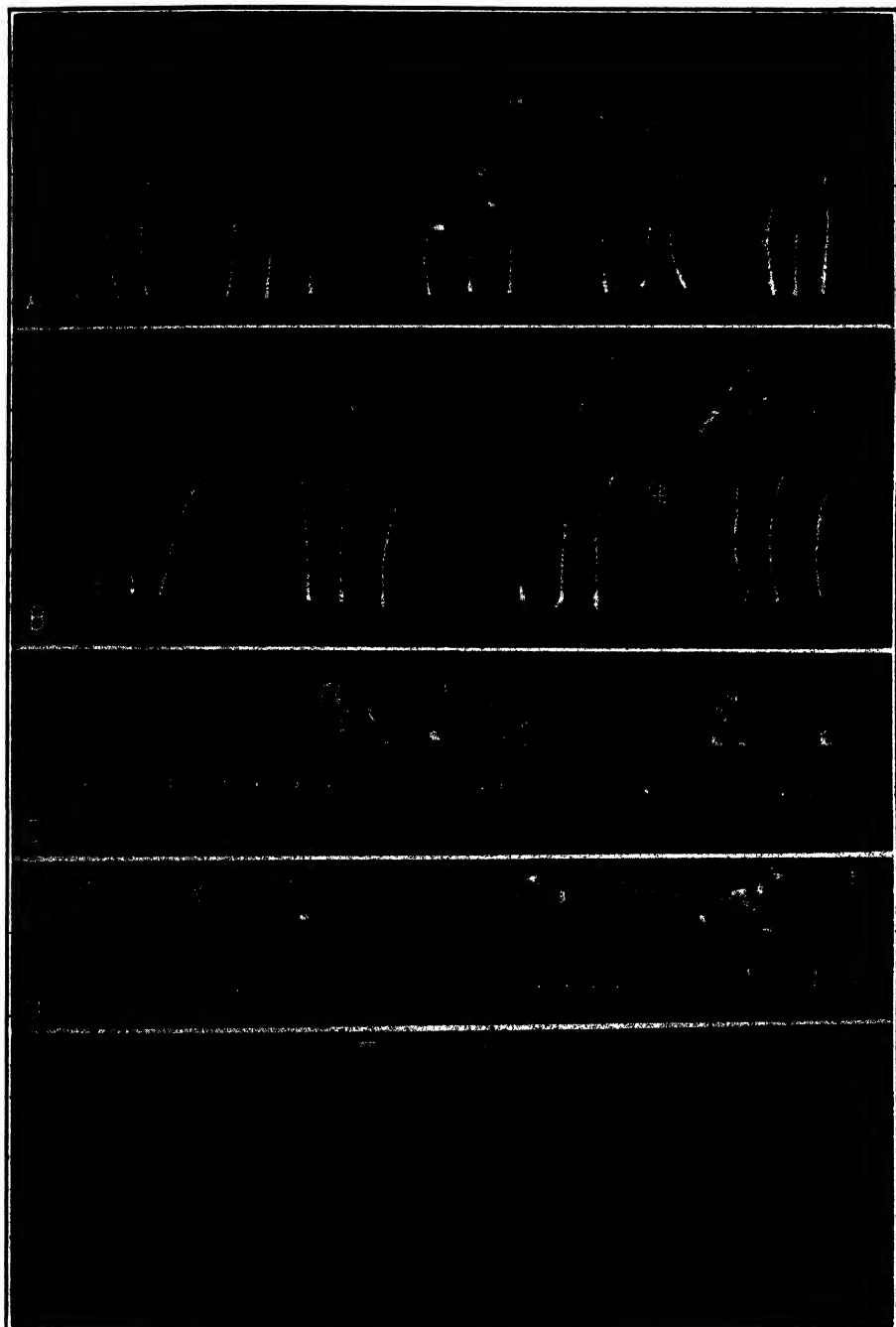


FIGURE 1. (For description see legend on opposite page.)

stream (14, 15). In the case of lanolin applications, however, transport appears to occur outside the transpiration stream as suggested by Hitchcock and Zimmerman (14, p. 470): "Responses at distant points resulting from lanolin treatments . . . appeared to be independent of transpiration. . . ."

Tests were then performed to see if in case of lanolin applications the chemical absorbed could move upward in a quantity sufficient to induce rooting of leaf cuttings made from the treated plant. Results of one such test are given in Table III. Treatment was made in accordance with the

TABLE III

RESULTS OBTAINED WITH TOMATO LEAF CUTTINGS AFTER THE STEM HAD BEEN TREATED WITH NAPHTHALENEACETIC ACID—LANOLIN PREPARATIONS. AVERAGE NUMBER OF ROOTS ON 4 CUTTINGS

Concentration mg. per g. lanolin	Test 1	Test 2	Average
0	2	4.2	3.1
5	0.2	0.5	0.3
10	1.5	7.2	4.3
20	11.2	21.5	16.3

description of methods; 96 hours after application of the growth substance, cuttings were taken from the region above the place of application, prepared and planted. Root counts were made 12 days after planting. The only concentration which was noticeably effective was 20 mg. per g. (Fig. 1 E). Other experiments showed a more pronounced effect of the treatment with 10 mg. per g. than is shown in Figure 1 E. With lanolin preparations it is difficult to obtain uniform results because the amount of response depends upon the amount of chemical absorbed and the penetration of the chemical into the tissues depends upon several factors—amount of lanolin applied, area covered by lanolin, number of hairs broken during application, permeability of the tissues, etc.

Cuttings taken from below the treated region formed a greater number of roots than the controls, indicating that the chemical moved downward. Bending of leaves and stems, and the swelling and proliferation of stems, below the treated region, also showed that the chemical had moved downward.

The next step was to determine through which channels the synthetic growth substances are transported. Hitchcock and Zimmerman (14) have shown that such chemicals are able to move through a ring of dead tissues either when a solution is supplied to the soil or injected into the base of petioles, or when lanolin preparations are used. Varying the region of treatment (above or below the dead segment) they demonstrated by bending responses that upward as well as downward movement takes place through dead tissues.

In the present tests tomato plants 9 and 11 inches tall with a dead segment of tissue in the middle of the stem (see Materials and Methods) each received 50 cc. of water containing 10 mg. of indolebutyric acid. Sixty-eight hours later three leaves were taken from the taller plants and two from the shorter, above the dead segment, and were planted. At the same time cuttings from plants with the dead segment but not treated were planted as controls. Root counts were made ten days later. The number and length of roots on cuttings from treated plants were exceedingly greater than on controls. Rooting above the base is an indication of a high concentration effect (Fig. 2).

Other tests have shown that the chemical is able to move upward even when applied to the killed base of tomato stems. In this case a solution of

TABLE IV

EVIDENCE OF TRANSPORT THROUGH DEAD TISSUES OBTAINED WITH TOMATO LEAF CUTTINGS. INDOLEBUTYRIC ACID WAS APPLIED TO THE SOIL OF PLANTS HAVING A DEAD SEGMENT OR THE KILLED BASE OF THE PLANT WAS DIPPED INTO A SOLUTION OF THE GROWTH SUBSTANCE. AVERAGE NUMBER OF ROOTS PER PLANT

Plant	Dead segment		Dead base	
	Control	Treated	Control	Treated
Long	3	22	4	23
Short	2	12.6	9	18.5
Average	2.5	17.3	6.5	20.7

indolebutyric acid of 5 mg. per liter was used. The killed region measured about 8 cm. and was dipped into the solution to a depth of 2 cm. (see Materials and Methods). Twenty-four hours later cuttings were made and planted. Cuttings taken from plants with killed bases in tap water for the same period were planted as controls. Root counts made eight days later revealed a much better rooting of cuttings from treated plants (Fig. 2). Table IV combines the results of the two experiments just described.

The ability of the chemical to move upward starting from dead tissues indicates that such a movement is a result of a force pulling from above rather than one pushing from below. It is interesting to mention here that Curtis (8) has shown that the downward translocation of products of photosynthesis is dependent upon the activity of living cells of the phloem. As he believes that inorganic compounds as well as sugars move through the phloem, also upward, he assumed that this "movement must be also dependent upon and controlled by living cells" (8, p. 164-165). Experiments with local chilling confirmed that assumption. Cooper (3) observed that local chilling reduced the transport of indoleacetic acid applied as lanolin preparations to the apex of leafless lemon cuttings. Cooper, however,

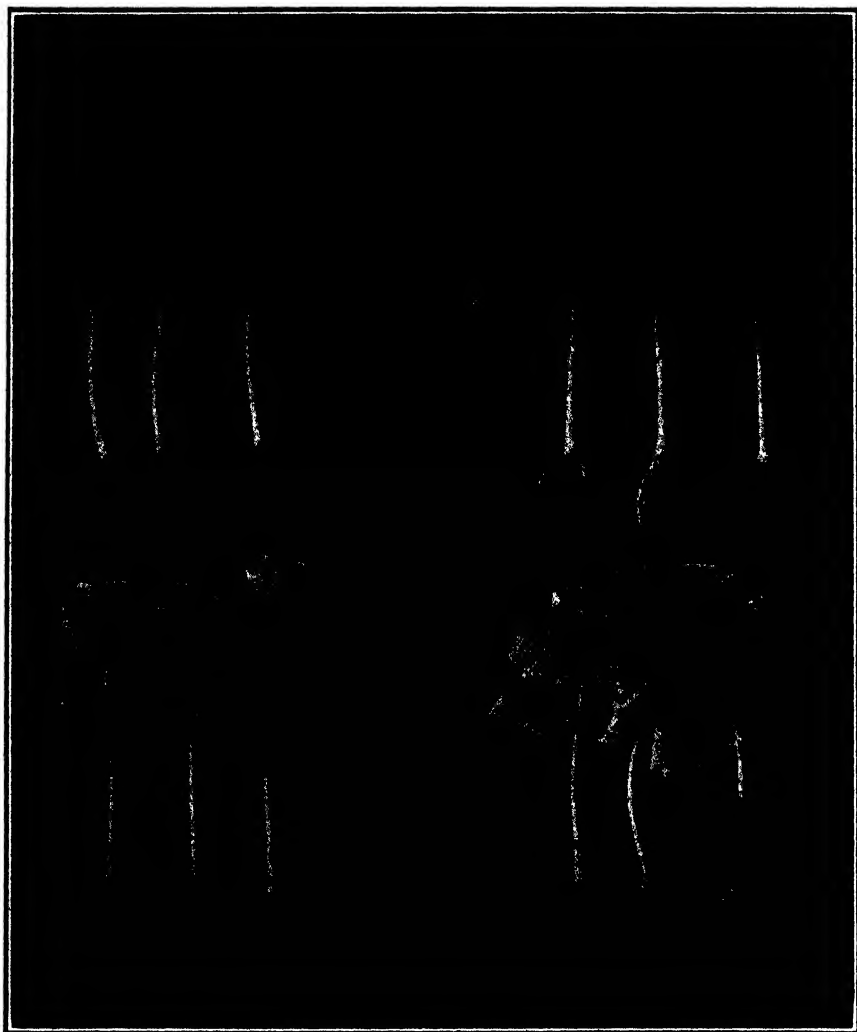


FIGURE 2. Conduction through dead tissues: tomato cuttings. Top row, plants with a segment of dead stem: left, control; right, treated with 10 mg. of indolebutyric acid in 50 cc. of water; cuttings made 68 hours after treatment remained 10 days in the rooting medium. Lower row, plants with the base of the stem killed, then dipped in water (control, left) or in a solution of indolebutyric acid (right) 5 mg. per liter for 24 hours; 8 days after planting.

did not have as controls cuttings chilled and not treated. It might be possible that chilling itself reduced the number of roots. This seems to have actually been the case, since the analysis of Cooper's figures shows that the chilled and treated cuttings formed fewer roots than those not chilled and not treated.

Since several papers have pointed out similarities and differences between modifications induced by synthetic growth substances and those induced by virus diseases (24, 25) it will be interesting to mention here that as far as the movement is concerned, there seems to be no analogy at all; the synthetic growth substances are able to move through dead tissues which hinder the movement of viruses according to Caldwell (1). This author showed that a ring of tissues killed with chloroform or by heat prevented the manifestation of the disease above the ring when the virus was inoculated below it.

Hitchcock and Zimmerman (14) observed that if a segment of wood inside the bark is removed from the stem of tobacco the downward movement of a natural hormone which inhibits the development of axillary buds stops and the buds below the region deprived of the wood begin to grow. The conclusion is, then, that this natural hormone moves through the wood and not through the bark. It is true, however, that such hormone failed to pass through a segment of dead tissues. But since in the case of lanolin preparations of synthetic growth substances the movement across dead tissues depended upon the succulence of the tissues, it was assumed that the failure of the natural hormone to move through dead tissues was due to its low concentration which did not allow it to reach the principal channels of longitudinal transport. Further experiments showed that synthetic hormones are absorbed from a solution by a slit portion of stem containing a fragment of wood but no absorption occurred when the wood was completely removed from the portion of stem immersed in the solution.

Once the fact was confirmed that living tissues are not necessary for the transport of synthetic growth substances, experiments were carried on to determine whether these substances move through the xylem or through the phloem. Leafless *Hibiscus* cuttings were prepared for these tests as shown in Figure 3 A and described under the heading Materials and Methods. Some of them had only bark in the base dipped into the solution, some only wood, and some both wood and bark. As explained above, a band of pure lanolin prevented a possible external rise of the chemical by "leakage." After being in the solution for a certain time these cuttings were re-cut above the lanolin band and planted. Table V shows the root counts for one such experiment. The efficiency of transport, as evidenced by the effect of the chemicals on rooting, was greatest for cuttings with wood alone; there was relatively little transport in the case of cuttings with both wood and bark and no transport in the case of bark alone. In this experiment,



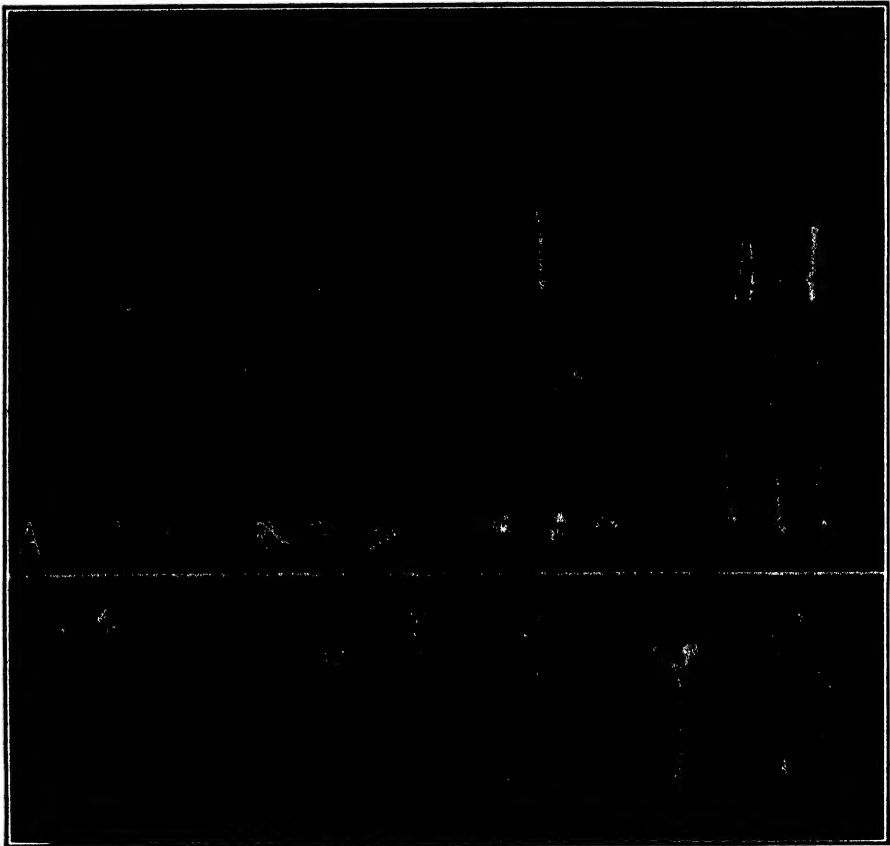


FIGURE 3. *Hibiscus* cuttings. A, upper row, indicates method of preparing cuttings before treatment; about 6 in. of the basal part was removed after treatment in every case; lower row indicates rooting of the different types of cuttings treated with indolebutyric acid 60 mg. per liter during 24 hours, 21 days after planting; from left to right, control; cuttings treated with both wood and bark present; with bark removed, and with wood removed. B, cuttings treated with indolebutyric acid 80 mg. per liter during 24 hours, 20 days in the rooting medium; each group of two cuttings shows control on the left; from left to right, wood removed from the base; bark removed; both bark and wood present; cuttings split in two halves for a distance of 2 inches and one-half removed; one inclined surface; and two inclined surfaces.

actually the cuttings with bark alone did not root as well as the controls. It would appear, therefore, that the removal of wood had been injurious to the cuttings, perhaps by a deficiency in the water supply during the treatment. The analysis of all the present tests showed, however, that this was not the case: in 8 out of 17 tests the controls rooted better than the treated cuttings with bark alone and 9 times the treated were better than the controls.

Looking at Table V one can see that the cuttings from which the bark had been removed rooted better than those with wood and bark. This surprising result appeared consistently throughout the experiments: 17 tests analyzed showed that the cuttings with wood alone ranked first 13 times;

TABLE V

RESULTS OBTAINED WITH HIBISCUS CUTTINGS AFTER TREATMENT WITH SOLUTIONS OF SYNTHETIC GROWTH SUBSTANCES. SOME CUTTINGS HAD ONLY WOOD, SOME ONLY BARK, AND SOME BOTH WOOD AND BARK WHEN THE BASES WERE TREATED. AVERAGE NUMBER OF ROOTS PER CUTTING

Treatment*	Non-treated controls	Wood	Bark	Wood and bark	Remarks
IB, 60 mg. per l., 48 hours	9.6	25.2	8	9.4	Averages of 5 cuttings; 24 days in rooting medium
NA, 60 mg. per l., 48 hours	7.4	10.6	8.4	10.2	
KIB, 60 mg. per l., 48 hours	11.6	26	0.3	12.6	Averages of 3 cuttings; 25 days in rooting medium
KNA, 60 mg. per l., 48 hours	7	11.3	0	12.3	
Average	8.9	18.2	4.1	11.1	

\* IB=indolebutyric acid; NA= $\alpha$ -naphthalenecetic acid; KIB=potassium indolebutyrate; KNA=potassium naphthalenecetate.

twice the cuttings with wood and bark were the best; once the cuttings with bark alone; and once the controls. The application of Friedman's method of ranks (12) which uses values of Chi Square showed that the differences in rooting observed among the four types of cuttings (controls, wood alone, bark alone, wood and bark) were significant at the 1 per cent level. The isolated comparison of each type of treated cuttings with the controls showed that treatment had been effective (significant at 1 per cent level) only on cuttings from which the bark had been removed. It is important to remember that the cuttings were recut after treatment before they were planted. The same concentrations used in the same period of treatment would be highly effective if the cuttings had not been recut.

These results are just the opposite of those obtained by Cooper (3). He ringed leafless lemon cuttings which he submitted to apical lanolin-indoleacetic acid treatment and observed that the chemical could not move

through the wood. When a bridge of bark was left some movement occurred.

Cooper, however, failed to perform the definite test: to take off a segment of wood leaving the bark alone to observe if movement could be detected. Furthermore, if we look at Cooper's figures, we can see that in all four non-treated controls, the non-ringed cuttings showed some roots whereas the ringed ones did not and when a longitudinal bridge of phloem was present, two out of three controls (one not shown in the table) showed some rooting. This strongly suggests that the ringing affected the cuttings under the conditions of Cooper's experiments. If the ring was not complete then the injury was not so pronounced.

Cooper also states that when three-fourths of an inch of the base of lemon cuttings was taken off after basal treatment with a solution, the effect of the treatment disappeared and retreatment of the base had little effect. He concluded, then, by admitting that the growth substance applied had not moved up to that distance and he suggested that the treatment determines a downward movement of some factor essential for rooting which accumulates at the base. Taking off the base depletes the cutting of that element and then retreatment can not be as effective as the initial treatment.

This idea of mobilization of another factor essential for root formation was also defended by Went (23) who proposed to call it "rhizocaline."

Hitchcock and Zimmerman (15) tested several species and obtained results which do not support such an hypothesis. All species did not respond alike. Retreatment of tomato cuttings from which 1 or 2 cm. of the treated base had been removed showed an additive effect. This and the fact that cutting off 1 or 2 cm. of the treated base did not eliminate, although it reduced, the effect of treatment, were taken as indicating that the applied growth substance (indolebutyric acid) had moved upward for a distance of more than 2 cm. In leafless grape cuttings the effect of treatment was removed by cutting off the base but retreatment was effective. Retreatment of leafless *Hibiscus* cuttings after removal of one-half or one inch of the treated base was also effective. Leafy cuttings of *Euonymus*, *Ilex*, *Rosa*, and *Viburnum* responded also to retreatment after three-fourths of an inch of the base had been cut off. Three segments of the base of *Ilex opaca* were successively removed after each 24-hour treatment so that at the end of the 72-hour total treatment, two and one-fourth inches had been cut off and yet no depletion of substances essential for root formation occurred.

In the present experiments with leafless *Hibiscus* cuttings, 6 cm. (more than 2 inches) of the treated bases were removed and the effect of treatment was evident in 13 out of 17 tests on cuttings without bark at the base. When cuttings had both wood and bark the removal of such an extension

of the basal segment eliminated the effect of treatment in more than 50 per cent of the cases. This means that when the bark was removed the chemicals moved upward for a distance of more than 6 cm. in an amount sufficient to induce root formation. When the bark was present, either the distance of upward movement was shorter or the amount of chemical transported was smaller.

It seems difficult to understand how substances essential for root formation could be induced to move downward and accumulated at the base, unless we admit that some stimulus moved upward in the xylem, since cuttings without bark at the treated base rooted even better than those with bark and wood.

If we admit that the applied growth substance moves upward, and acts by itself or as a stimulus to determine a redistribution of hormones already present, then the facts here mentioned can be understood. The bark in some way prevents the entrance of chemicals. Since the cross section of the wood is practically the same in cuttings with or without bark, it would appear that some penetration occurs along the lateral walls when the bark is removed; when the bark is present this lateral movement is prevented by its impermeability.

That the chemical can penetrate through the lateral walls was shown by the following experiment: some of the cuttings from which the bark had been removed at the base for a distance of 5.5 cm. were coated with paraffin applied laterally to the wood; other cuttings were coated with paraffin at the base. In the first case only the base was in contact with the solution, in the second only the lateral walls. In both cases the rooting was better than in control cuttings. The two treatments did not show much difference.

In view of these results the following explanation is proposed. When the bark is removed a greater surface of penetration is exposed to the solution; the number of vessels which might carry the chemical upward is always the same but some of them may be plugged near the base and free a little above; if the bark is removed these vessels can also transport the chemical which enters sidewise above the plugged part, while if the bark is present these vessels are not used.

If this explanation is correct then the greater the surface exposed to the solution, the more efficient will be the treatment. Some experiments were performed to test this. Cuttings were prepared as shown in Figure 3 B, in some of them the wood was taken off for a distance of 5.5 cm. from the base; other cuttings were left with only wood in this part; others remained with wood and bark but had different cut surfaces; some had a transverse cut surface; others had a long (5.5 cm.) longitudinal and half a transverse cut surface (the cutting was split in two halves and one-half was taken off for 5.5 cm.); others had one inclined surface and finally others had two inclined surfaces, for the same distance. A solution of indolebutyric acid of

80 mg. per liter covered the bases of these cuttings for about 2 cm. during 24 hours. Cuttings were planted without being recut. Upward movement of the growth substance did not occur in cuttings with bark alone, but occurred in all other cases. When the wood was left alone roots were induced to grow 5.5 cm. above the base, where cambium was present. The number of roots was a little greater in the lot with a transverse cut surface but the roots were larger in the other lots and they were formed all over the ridges of the cut surfaces thus indicating that upward movement actually occurred since only the lower 2 cm. were dipped into the solution.

Although the present tests were not sufficiently extensive to show definitely that the penetration of the growth substances is proportional to the surface exposed to treatment, the results indicated this to be the case. Experiments prepared in the same way as the one just described, in which the cuttings were recut before planting to provide the same surface for rooting, showed a slight difference in favor of the greatest surface.

#### SUMMARY

1. Synthetic growth substances applied as solutions to the soil of potted tomato and *Cleome* plants can be absorbed and transported upward in an amount sufficient to induce root formation on leaf cuttings taken some time after treatment. Indolebutyric, naphthaleneacetic, indoleacetic, and 2,4-dichlorophenoxyacetic acids have been tested.

2. When lanolin preparations of synthetic growth substances are applied to the stem of tomato plants, the amount of chemical absorbed and transported upward to the leaves may also be enough to induce root formation.

3. The upward movement of synthetic growth substances is not stopped by a ring of dead tissues in the middle of the stem. These substances can also move upward when applied to the killed base of tomato stems. In both cases roots were induced above the base of petioles which is evidence of a high concentration effect. Thus the movement is independent of the activity of living cells.

4. No movement of synthetic growth substances was detected on leafless *Hibiscus* cuttings when a segment of wood was removed for a distance of 5.5 cm. from the base and the bark alone was dipped into the solution for a distance of 2 cm. When the bark was removed and the wood was left alone for the same distance the movement was even more intense than when both wood and bark were present. In all these cases a segment of the base of about 6 cm. was cut off after treatment. A band of pure lanolin prevented a possible external rise by capillarity. Indolebutyric and naphthaleneacetic acids and the potassium salts of both were employed. The effect of treatment was evident on cuttings with wood alone but not on cuttings with wood and bark, when all treatments were statistically analyzed

together. The bark, then, not only did not transport but also in some way decreased the amount of growth substance transported. It is suggested that when the bark is removed some chemical enters through the lateral walls of the wood and moves upward through vessels which, being plugged at the base, would not be utilized if the impermeable bark were present at the region exposed to the solution.

5. The present results support the view of upward translocation of solutes in the xylem.

#### ACKNOWLEDGMENT

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## EFFECTS ON MICE OF A DIET CONTAINING METHYL ESTER OF $\alpha$ -NAPHTHALENEACETIC ACID

NANCY FINCH AND ALBERT HARTZELL

Guthrie (9) showed that the vapor of the methyl ester of naphthaleneacetic acid inhibited the sprouting of potato tubers. Denny (4) found that the amount of the chemical needed to inhibit the sprouting of tubers from October to April at a temperature of about 10° to 15° C. ranged from about 25 mg. to 100 mg. of the methyl ester of  $\alpha$ -naphthaleneacetic acid per kilogram of tubers. He also showed that tubers exposed over that period to the methyl ester at the rate of 100 mg. per kg. of tubers contained approximately 5 mg. of the chemical per kg. of tissue, of which amount about 80 per cent was in or on the skin of the tuber.

A review of the literature on the effects of naphthaleneacetic acid and related compounds on animals follows.

Acute intraperitoneal toxicity of naphthaleneacetic acid in mice has been shown by Anderson, Shimkin, and Leake (1) at a concentration of 100 mg. per kg.

Elliot (7) observed a marked acceleration in the growth of protozoa (*Euglena gracilis*) using 3-indoleacetic acid,  $\beta$ -3-indolepropionic acid, and  $\gamma$ -3-indolebutyric acid at pH 5.6 and at concentrations of 1:1,000,000 and 1:10,000,000.

Narat and Chobot (11) found that rats injected with potassium naphthaleneacetate and potassium indolebutyrate were not accelerated in growth rate by either chemical. The effect on local cell growth, however, was pronounced. Both chemicals shortened the healing time of artificially produced burns 35 per cent.

That the effectiveness of pituitary gonadotropic extract is increased in the immature rat after admixture of indolebutyric acid and  $\alpha$ -naphthaleneacetic acid was observed by Breneman (2). The action of the plant growth hormone when used separately was questionable.

Indole-3-acetic acid inhibited normal respiration of both sarcomas and carcinomas in mice, according to Robinson and Taylor (12). Tuboi (13) also reports inhibition of these carcinomas with this compound. It has been pointed out by Kögl, Haagen-Smit, and Tönnis (10), that certain indole compounds occur in carcinomatous tissue in concentrations twice that of normal tissue.

Dye, Overholser, and Vinson (6) tested  $\alpha$ -naphthaleneacetic acid, amide of  $\alpha$ -naphthaleneacetic acid, maleic acid, and succinic acid. Rats were injected subcutaneously with each of the substances beginning at the



seventh day of age with a dose of 0.5 mg. (in sesame oil). The dosage was increased until each rat was receiving 2.0 mg. daily until 60 days of age. The control rats received sesame oil. Larger doses (5 to 10 mg.), continuing over a period of three weeks or more, produced toxic symptoms in the young rats. Some of the rats died and otherwise showed retardation of growth and patchy distribution of hair. They conclude that no one of the six compounds produced significant effects on growth and the rate of development of albino rats, nor did they exhibit any estrogenic properties in the doses (2 mg.) used. Negative results were also obtained with chick embryos with injections of 0.05 cc. of a 1:1000 solution of each of the substances.

### MATERIALS AND METHODS

Tubers treated in 20-bushel bins with 100 mg. of methyl ester per kg. of tubers (from October 1941 to April 1942) were used for feeding tests with mice to determine whether such tissue was toxic. With some of the lots of mice the chemical was added to the treated potato tissue in increasing amounts until one lot received approximately 90 times as much chemical as is estimated to be present in potato tissue treated with the chemical.

*Diet.* The basic diet used for the first seven and one-half months consisted of 150 g. of whole potatoes, 274 g. of powdered milk, 10 g. of yeast, 5 g. of celluration, and 350 g. of water. For the methyl ester series, potatoes that had been treated with the ester were used. These, from previous tests, were considered to contain methyl ester in a concentration of about 5 mg. per kg. In further lots, additional methyl ester (dissolved in cod liver oil) was added to give a quantity equivalent to 150 g. of potato tissue, containing 50 mg. per kg., 150 mg. per kg., and 450 mg. per kg. Calculated in another way, the diets contained 0, 0.95, 9.5, 29, and 86 mg. of the ester per kg. of the diet. Later, in order to avoid feeding so much potato tissue, since it is not a natural food for mice, and to improve the diet in other respects, instead of using 150 g. of whole potato tissue, 75 g. of thick peel were used, together with 30 g. of casein and 45 g. of wheat flour. The amount of powdered milk was increased 11 g. When this change was made the amounts of methyl ester added were such as to maintain the methyl ester contents of the diets at essentially the same levels per unit of diet as previously used.

The diet was made up weekly, stored at 5° C., and portions were removed daily from the refrigerator. The amount of meat, pellets, and lettuce eaten over the week-end could only be estimated. Therefore, the methyl ester diet was changed in March 1943 to include only week days, leaving the food consumed over the week-end entirely separate and not included in the formula.

*Life span.* An experiment was set up to observe the possible effects of continuous feeding of methyl  $\alpha$ -naphthaleneacetate to mice, beginning at an age of approximately two months.

One hundred female and ten male Swiss albino mice were obtained from Columbia University, New York, N. Y. The females were segregated ten to a cage and the males one to a cage. They were fed the experimental diet on week days in the following dilutions: (A) 2 cage lots, 20 mice, fed diet with potato not treated with methyl  $\alpha$ -naphthaleneacetate and without the added chemical; (B) 2 cage lots, 20 mice, were fed a diet with potato treated with the methyl ester and no added ester; (C) 2 cage lots, 20 mice, were fed a diet similar to the above but with 10 times the amount of methyl ester believed to be present in the potato tissue treated with methyl ester; (D) similarly, 2 cage lots, 20 mice, were fed a diet with 30 times the amount of methyl ester; (E) similarly, 2 cage lots, 20 mice, were fed a diet with 90 times the amount of methyl ester.

The mice were weighed by cage groups at intervals of two weeks. All these animals were kept until the end of their natural lives. Whenever possible, autopsies were performed on each animal at the time of death.

*Breeding.* Four females were selected at random from each cage lot for breeding on October 28, 1942. These were bred simultaneously to the males which had been fed corresponding diets and dilutions of methyl ester. After two and one-half weeks the females were segregated. In every case in which a litter was not produced, the female was rebred.

Young were weighed at birth, 14 days, and 21 days, by litter lots. When weaned, young were combined in groups of approximately the same age. These lots were fed like the original groups and observed until litters again began to be produced. This date was considered the maturity date. At this stage males and females were segregated. When all lots of young of a single *diet dilution* were mature the males and females were again combined. After two weeks the females were isolated. Fifty young of the resulting litters were kept after weaning to make up generation II, discarding principally males. Generation I mice not used for further tests were discarded due to lack of cages because of shortage of vital metals which were not obtainable due to the war. Generation II mice were fed and checked for maturity similarly to generation I, rebred, and discarded as soon as the young were weaned. The same procedure was followed for generations III and IV, except that mice of the fourth generation were not bred. All mice in both phases of the experiment were fed identically, all changes in the diet being applied to all mice. Graphs of average weights, etc., were kept concurrently. The generation groups of mice were weighed every two weeks until discarded.

*Analysis of organs.* Organs of five mice which had been fed methyl ester

in a dilution of 10 times and 90 times the amount believed to be present in the potato tissue treated with methyl ester were tested by Dr. F. E. Denny (4) for presence of the ester.

*Paratyphoid tests.* Female mice which were bred were tested<sup>1</sup> for the presence of paratyphoid bacilli in the feces by the brilliant green agar method (3).

### RESULTS

The three criteria by which the relative effect of the diets containing methyl ester were determined are growth, reproduction, and longevity.

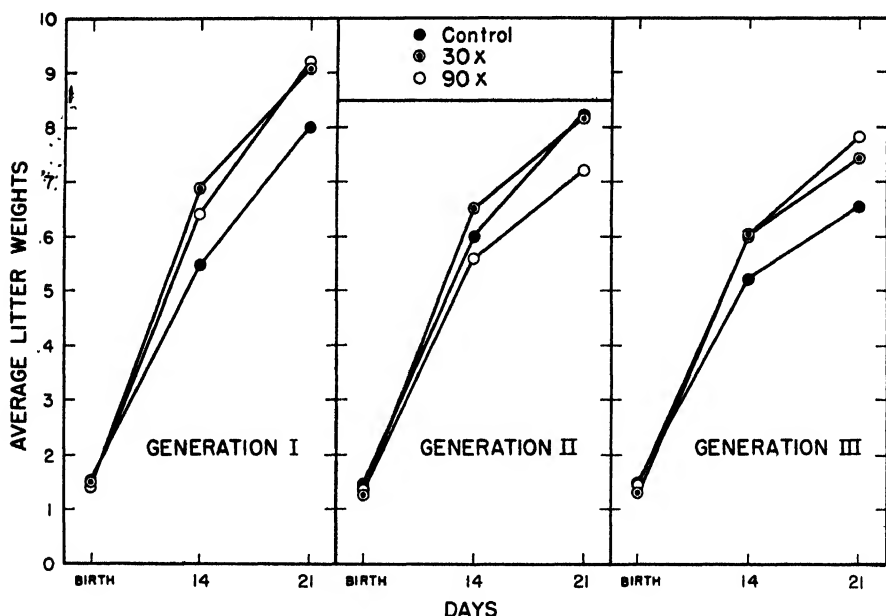


FIGURE 1. Growth curves of litters from birth to age of 21 days for females fed the following amounts of methyl ester of  $\alpha$ -naphthaleneacetic acid: 30 times and 90 times the amount estimated to be in treated potato, as compared with control.

*Growth.* Rate of growth of the young mouse from birth to weaning on the twenty-first day was determined. Litters born of females that had been fed continuously throughout the experiment 30 times and 90 times the amount of methyl ester found in treated potatoes were weighed at birth, 14 days, and 21 days. A comparison was made of the average weight of these young with the control. The results are shown graphically in Figure 1. There appears to be no significant difference between treatments in any one generation or between the three generations observed. A number of

<sup>1</sup> The writers are indebted to Dr. C. A. Slanetz of Columbia University, New York, N. Y., for paratyphoid tests and guidance in the medical phases of this work.

fourth generation mice were reared which appeared to be normal in growth response but no weighings were made.

**Reproduction.** The vitality of the adult was measured by the ability of the female to bear and rear young. It will be noted in Table I that the number of young born to females receiving 30 times and 90 times the amount of methyl ester in treated potatoes compares favorably with the control lots. All females bred bore young and so far as could be determined the males fed methyl ester continuously throughout the experiment showed no evidence of sterility. The number of young reared per litter ranged from 3 to 12 individuals.

TABLE I

LITTER RECORD FOR FEMALES IN COMPARISON OF DIETS CONTAINING METHYL ESTER OF  $\alpha$ -NAPHTHALENEACETIC ACID

Generation I						Generation II						Generation III					
Control		30 X*		90 X**		Control		30X		90 X		Control		30 X		90 X	
No. born	No. survived	No. born	No. survived	No. born	No. survived	No. born	No. survived	No. born	No. survived	No. born	No. survived	No. born	No. survived	No. born	No. survived	No. born	No. survived
9	7	11	9	9	4†	7	7	9	9	8	8	8	8	9	8	7	7
9	9	8	7	10	9	6	6	11	6	9	9	9	9	9	9	7	7
8	7	8	7	9	8	10	7	7	7	10	10	8	8	8	6	6	6
7	7	6	6	10	9	9	9	10	8	10	10	8	8	9	7	6	6
8	8	4	5	5	5	12	9	9	9	5	5	8	8	7	7	7	6
7	7	8	8	9	9	9	9	10	8	8	8	5	5	3	3	5	5
7	7	—	—	5	5	8	8	10	10	10	7	9	9	7	7	9	9
—	—	—	—	3	3	8	8	8	8	4	4	—	—	7	7	8	8
55	52	46	41	60	52	69	63	74	65	64	61	55	55	59	54	55	54

\* Quantity of methyl ester fed in cod liver oil approximately 150 mg./kg. of potato tissue in diet.

\*\* Quantity of methyl ester fed in cod liver oil approximately 450 mg./kg. of potato tissue in diet.

† Three died, two eaten by female, four survived.

The totals in Table I show a survival of 170 (i.e., 52+63+55) out of 179 for the control lots, and 160 out of 179 for the lots receiving the 30-times dose, and the odds against obtaining this result on the basis that the chemical had no effect are about 10 to 1. However, this result may not be important since there was no difference in the survival rate between the lots receiving the 90-times dose and the controls.

There appears to be no evidence also that there is a cumulative effect from one generation to another due to treatment.

**Longevity.** The results of the life span experiment are shown graphically in Figure 2. It will be observed that the mice in all treatments exhibit nor-

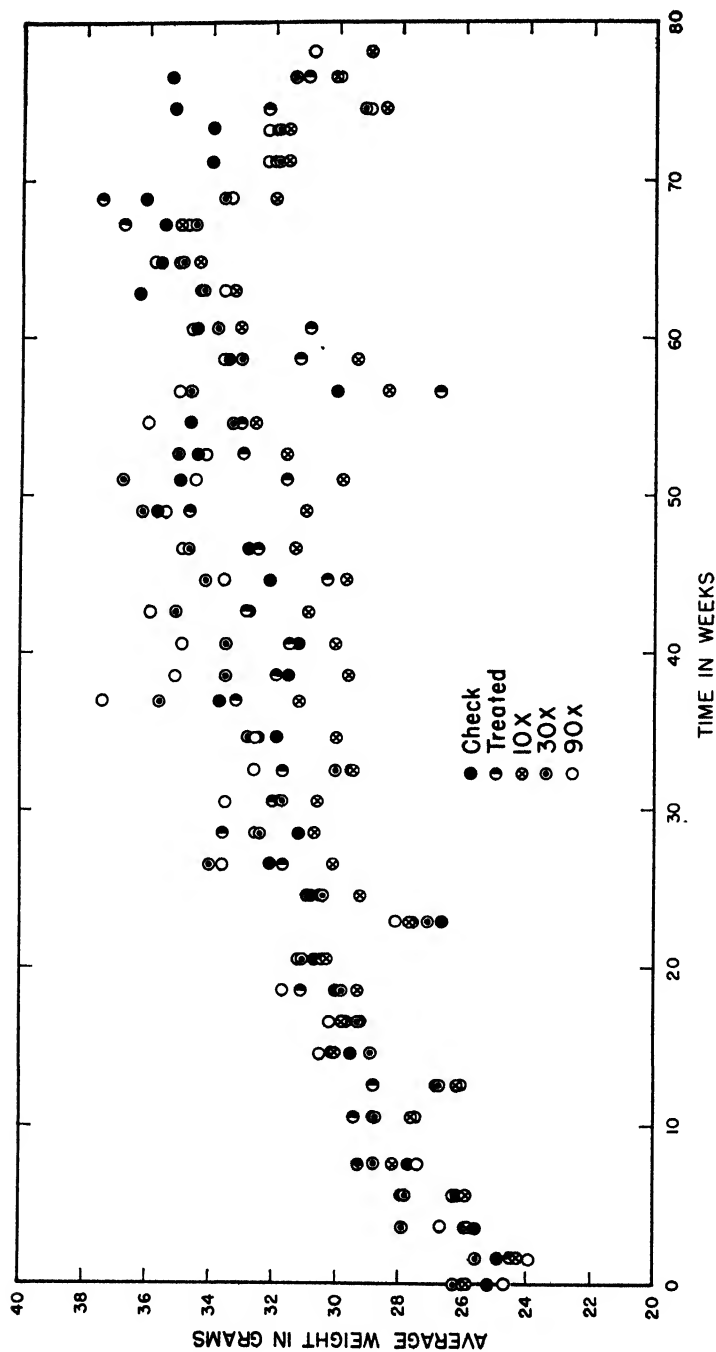


FIGURE 2. Curves for average weight of mice fed a diet comprising treated potato containing methyl ester of  $\alpha$ -naphthaleneacetic acid, and 10 times, 30 times, and 90 times the amount estimated to be in treated potato, as compared with control.

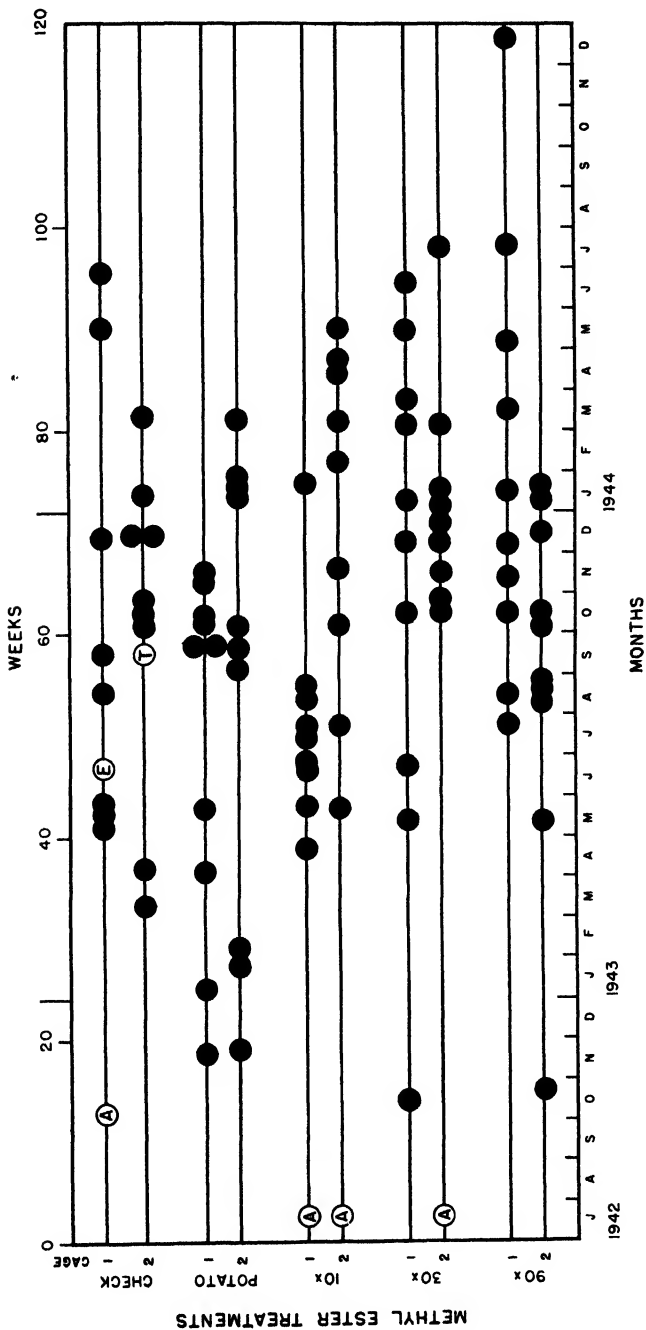


FIGURE 3. Mortality chart of mice in the adult life span feeding experiment: solid circle = normal; A = accidental; E = escaped; T = tumor.

mal weight curves when compared with the control. The general appearance and vigor of the mice was normal throughout the test in both control and treated lots. The percentage survival at the end of seven months was 90 per cent, which compares with 83.6 per cent survival for a normal herd of mice reported by Greenwood and others (8) for the same period.

The number of mice surviving at the end of one year was 13 in the control and 60 in the treated lots. A comparison of the deaths in any treated

TABLE II

TYPICAL GROSS PATHOLOGIC FINDINGS AT AUTOPSY OF MICE\* WHICH DIED IN THE METHYL ESTER  $\alpha$ -NAPHTHALENEACETIC ACID EXPERIMENT

Organs	Life span experiment					Generations I to IV		
	Control	Treated**	10 X	30 X	90 X	Control	30 X	90 X
Brain	Normal	Occasionally congested	Normal	—	Normal	Normal	Normal	—
Thyroid	Not enlarged	Not enlarged	Not enlarged	Not enlarged	Not enlarged	Not enlarged	Not enlarged	—
Lungs	Moderately distended, congested and hemorrhagic. Bronchopneumonia	Moderately distended, hemorrhagic, occasionally fatty degeneration	Congested, pneumonic areas	Congested, hemorrhagic. Bronchopneumonia	Moderately distended, congested and hemorrhagic. Bronchopneumonia	Moderately distended, pneumonic areas	Congested and hemorrhagic	Congested and hemorrhagic
Heart	Normal	Normal	Normal	Occasionally clotted	Normal	Normal	Normal	Normal
Spleen	Normal	Occasionally enlarged	Normal	Normal	Normal	Normal	Normal	Normal
Liver	Normal	Normal	Normal	Normal	Nutmeg	Normal	Normal	Normal
Gall bladder	Occasionally distended	Not distended	Occasionally slightly distended	Occasionally distended	Occasionally distended	Occasionally distended	Normal	Normal
Stomach	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Intestine	Occasionally injected†	Occasionally injected†	Normal	Congested	Congested	Normal	Normal	Normal
Kidney	Normal	Normal	Normal	Normal	Congested	Normal	Normal	Normal

\* Based on 40 individuals.

\*\* Potatoes treated with methyl ester under conditions for inhibiting sprouts and containing the amount of methyl ester normal under such conditions and thought to be approximately 5 mg./kg.

† Refers to blood vessels.

lot with the control shows no differences that can be considered significant. The mortality of the mice throughout the life span experiment is shown graphically in Figure 3.

Owing to the fact that among adult male mice the more aggressive individuals will severely injure or even kill less aggressive though normal adult males when caged together, adult male mice were not found to be satisfactory for studies of comparative average gain in weight. In this

investigation, therefore, all average gains in weight of adult mice reported are based on results obtained with females.

The two adult male mice used in each experiment lived the following number of weeks when fed the same concentrations of methyl ester as the females: treated potato, 70 and 112 weeks; 10 times the concentration found in treated potato, 28 and 18 weeks; 30 times concentration, 46 and 40 weeks; 90 times concentration, 88 and 24 weeks; control, 70 and 56 weeks.

*Pathology.* Autopsies (Table II) were performed immediately after death, if possible. If examinations could not be made immediately, the carcasses were kept at 5° C. for periods ranging from 16 to 48 hours until autopsies could be made. The chief cause of death was pneumonia as indicated by hemorrhagic lesions and distension of the lungs (5). The lungs of several mice examined showed multiple small nodules resembling tubercles, indicating pseudotuberculosis. A number of animals during the fore part of the life span experiment had scaly skin and marginal necrosis of the ears, signs that are usually associated with catarrh. Only two of the 40 individuals examined had enlarged spleens. There was only one case of breast carcinoma. None of the mice autopsied showed evidence of gross pathologic change that could be attributed to methyl ester of  $\alpha$ -naphthaleneacetic acid. Microscopic preparations were made of thyroid, lung, heart, spleen, liver, and kidney tissue of both treated and control animals for a possible future histological study.

Analyses of organs for methyl ester of  $\alpha$ -naphthaleneacetic acid from mice fed 10 and 90 times the amount believed to be in potato tissue were negative.

The paratyphoid tests of females which were bred also were negative.

#### SUMMARY

Mice fed a diet throughout their adult life span containing methyl ester of  $\alpha$ -naphthaleneacetic acid in treated potato, and concentrations added to the diet of 10, 30, and 90 times the amount believed to be present in treated potato, exhibited normal weight curves when compared with the controls. The methyl ester present in treated potato was estimated at approximately 5 mg. per kg.

There was no evidence of an accumulative effect upon the survival of the mice even with the highest amount of the methyl ester used or that the methyl ester either increased or diminished the mortality rate.

The general appearance of the treated mice was normal when compared with the controls.

Litters born of females that had been fed continuously throughout the experiment 30 times and 90 times the amount of methyl ester found in treated potato, showed no significant difference in number of young born,



per cent reared, or rate of growth from birth to the age of 21 days, either in the first, second, or third generation in the highest concentration (90-times dose) when compared with the controls. Percentage survival in the 30-times dose was less than for the 90-times dose or the controls, and the odds against obtaining this result on the basis that the chemical had no effect are about 10 to 1 and are considered not significant in view of the fact that higher amounts of chemical produced no effect. In other respects the mice fed the 30-times dose were similar in response to the controls.

Autopsies of mice that died during the course of the experiments showed no gross pathologic change that could be attributed to the methyl ester.

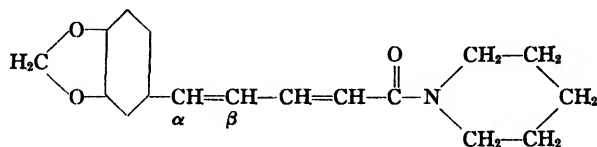
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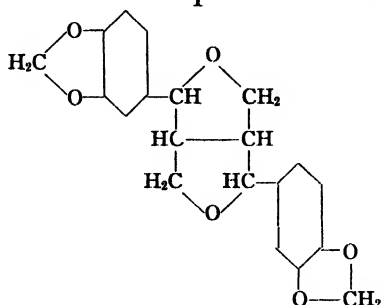
# SOME COMPOUNDS CONTAINING THE 3,4-METHYLENEDI-OXYPHENYL GROUP AND THEIR TOXICITIES TOWARD HOUSEFLIES

MARTIN E. SYNERHOLM AND ALBERT HARTZELL

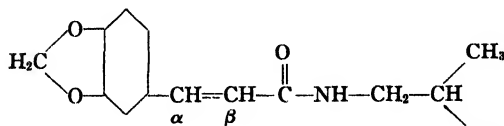
There are some naturally occurring substances containing the methylenedioxyphenyl group which are known to be toxic toward houseflies. Outstanding among these are piperine (I) (7, 8, 12), sesamin (II), (4, 6), and fagaramide (III) (5). The latter two appear to have little toxicity of their own, but enhance to some extent the efficacy of pyrethrum sprays when



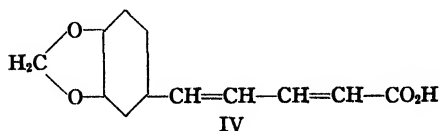
I



II



III



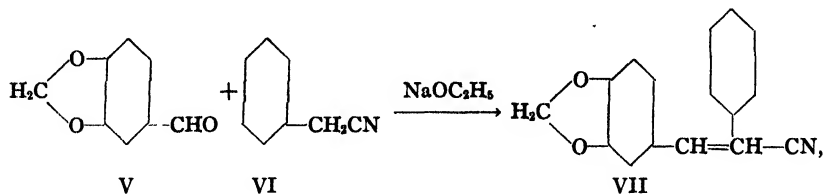
IV

used against houseflies. Piperine, as well as several closely related amides and esters of piperic acid (IV), have been shown (12) to possess exceptional toxicity when used alone as well as in addition to pyrethrins in housefly sprays.

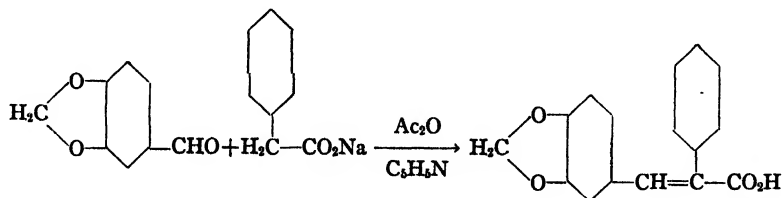
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This paper constitutes a report on additional 3,4-methylenedioxyphenyl compounds which have been synthesized and tested as fly sprays in this laboratory.

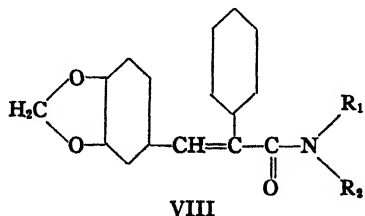
Piperonal (V) is a convenient starting material for the preparation of certain types of 3,4-methylenedioxyphenyl compounds having  $\alpha,\beta$ -unsaturation in the side chain, a structure present in piperine and its analogs and in fagaramide. When piperonal is condensed with benzyl cyanide (VI):



$\alpha$ -phenyl- $\beta$ -piperonylacrylonitrile (VII) is formed, and has been found to be an effective synergistic agent with pyrethrins against houseflies. Hydrolysis of this nitrile to the corresponding acid by refluxing in amyl alcoholic sodium hydroxide has been reported in the literature (2). This acid has now been prepared directly by condensing piperonal with sodium phenylacetate by the Perkin condensation using acetic anhydride and pyridine:

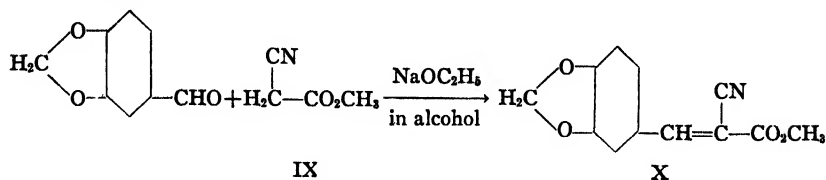


The acid obtained may be converted through its chloride to amides of the

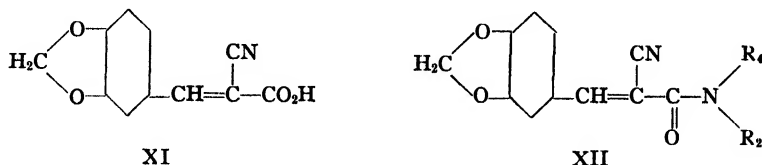


type VIII, where the groups  $\text{R}_1$  and  $\text{R}_2$  are determined by the amine used in their preparation. Five amides of this type have been prepared. Their effects on houseflies are reported in Table I.

When piperonal is condensed with methyl cyanoacetate (IX):

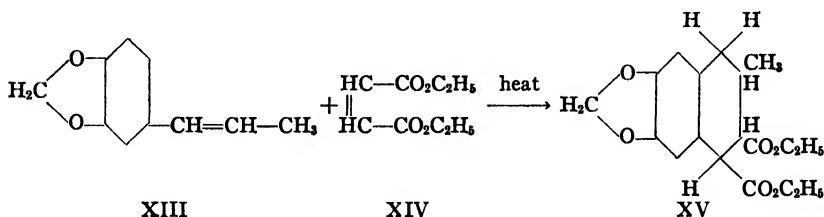


and the resulting ester is saponified and converted through the acid (XI) to amides (XII), these are found to possess high toxicity toward houseflies when used with pyrethrins.



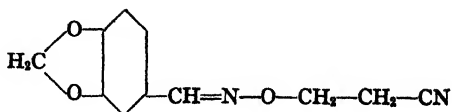
These amides are reported in Table II.

Hudson and Robinson (9) have shown that isosafrole (XIII) will condense with diethyl maleate (XIV) in a Diels-Alder type condensation to form diethyl-6,7-methylenedioxy-3-methyl-1,2,3,4-tetrahydronaphthalene-1,2-dicarboxylate (XV).



Eight esters of maleic acid have been condensed with isosafrole according to the procedure of Hudson and Robinson. The products formed are assumed to be of the type XV as was proved by these workers for the ethyl ester. The condensation products are viscous oils which could not be distilled under the vacuum obtainable. None of them could be made to crystallize. They were partially purified by repeated extraction with petroleum ether (30 to 60°). The results obtained with these esters are reported in Table III.

The addition of oximes of aldehydes and ketones to double bonds activated by carbonyl or cyano groups has been reported by Bruson and Riener (3). This reaction has now been applied to piperonaldoxime and acrylonitrile in the presence of sodium hydroxide to form an oximino ether having, according to Bruson's assumption, the formula:



Analysis for nitrogen is in fair agreement with that calculated for a compound of this composition. Peet-Grady tests run on this compound show it to be among the more active of those compounds possessing the methylenedioxyphenyl group that were tested. The results of these tests are reported in Table IV.

#### MATERIALS AND RESULTS

Piperonal, benzyl cyanide, phenylacetic acid, methyl cyanoacetate, isosafrole, ethyl maleate, *n*-propyl maleate, *n*-butyl maleate, cyclohexyl maleate, benzyl maleate, cyclohexylamine, isoamylamine, di-*n*-butylamine, butylamine, and piperidine were obtained from Eastman Kodak Co.

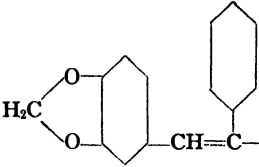
*α-Phenyl-β-piperonylacrylonitrile* (1, p. 284). Twelve grams of benzyl cyanide and 15 g. of piperonal were mixed in a 250-ml. Erlenmeyer flask. One gram of sodium was dissolved in 15 ml. of absolute alcohol. The alcoholic solution was added to the mixture in portions of 2 to 3 ml. followed by vigorous shaking and cooling under the tap. When the addition was complete, the mixture was diluted with 25 ml. of alcohol, filtered, and washed well with water. The solid after recrystallization from alcohol weighed 19 g. and melted at 121° (lit. 122°).

*α-Phenyl-β-piperonylacrylic acid*. Sodium phenylacetate was prepared by mixing a solution of 50 g. of phenylacetic acid in 100 ml. of alcohol with a solution of sodium ethylate prepared by dissolving 9 g. of sodium in 100 ml. of alcohol. The precipitated sodium salt was filtered, washed with alcohol, and dried at 100°. Fifty grams of sodium phenylacetate, 16 g. of powdered potassium carbonate, 2 ml. of pyridine, 48 g. of piperonal, and 45 ml. of acetic anhydride were mixed thoroughly in a one-liter round-bottomed flask. The flask was equipped with a reflux condenser and immersed in an oil bath previously heated to 180°. The mixture was kept in the bath, which was held at 180 to 190°, for two hours. After cooling, it was poured into 1200 ml. of water. The lumps were broken up by warming and stirring the mixture, which was then made alkaline by the addition of 10 per cent potassium hydroxide and filtered. The filtrate was extracted with ether, then acidified with dilute hydrochloric acid. The precipitated acid was filtered and recrystallized from glacial acetic acid. The yield was 35 g. of material melting at 230 to 231°. This material has been prepared by Bodroux (2), who reported 233° as the melting point. The ether extract, after being shaken with aqueous sodium bisulfite to remove unchanged piperonal, was concentrated and diluted with petroleum ether, whereupon 5 g. of 3,4-methylenedioxy stilbene precipitated, m.p. 90 to 91°, resulting

from decarboxylation of the  $\alpha$ -phenyl- $\beta$ -piperonylacrylic acid. The methylenedioxy stilbene showed very little toxicity toward houseflies.

*Amides of  $\alpha$ -phenyl- $\beta$ -piperonylacrylic acid.* A mixture of 5 g. of the acid, 10 ml. of thionyl chloride and 50 ml. of benzene was warmed on a

TABLE I  
TOXICITIES TOWARD HOUSEFLIES OF SOME DERIVATIVES OF  
 $\alpha$ -PHENYL- $\beta$ -3,4-METHYLENEDIOXYPHENYLACRYLIC ACID DERIVATIVES

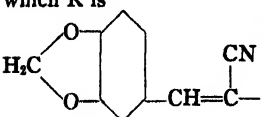
Formula of compound in which R is	M.p. °C. (uncorr.)	% Nitrogen		G. per 100 ml.	G. pyrethrins per 100 ml.	% Kill	% Knock-down*	O.T.I. % kill
		Calcd.	Found					
								
$\text{R}-\overset{\text{O}}{\parallel}{\text{C}}-\overset{\text{H}}{\text{N}}-\text{C}_6\text{H}_{11}$ (N-Cyclohexyl amide)	106-7	4.01	4.14	0.5 0.5 0.25	0.025 — 0.025	91 74 83	89	51 50 51
$\text{R}-\overset{\text{O}}{\parallel}{\text{C}}-\overset{\text{H}}{\text{N}}-n-\text{C}_4\text{H}_9$ (N- <i>n</i> -Butyl amide)	Oil	Not purified		0.5	0.025	74		37
$\text{R}-\text{C}-\text{N} \begin{matrix} n-\text{C}_4\text{H}_9 \\ n-\text{C}_4\text{H}_9 \end{matrix}$ (N-Di- <i>n</i> -butyl amide)	Oil	Not purified		0.5	0.025	74		37
$\text{R}-\overset{\text{O}}{\parallel}{\text{C}}-\text{N}(\text{CH}_2)_5$ (Piperidide)	124-5	4.17	4.26	0.5 0.5 0.25	0.025 — 0.025	78 31 49	91	41 37 45
R-CN (Nitrile)	121 (lit. 122)			0.5 0.5 0.25	0.025 — 0.025	88 30 72	49	51 57 56
Pyrethrins alone				—	0.025	19	93	48

\* In every case in which pyrethrins were used, the knockdown was greater than 90 per cent after 10 minutes.

steam plate until clear. After cooling, 50 ml. of petroleum ether was added; the acid chloride was filtered and washed with petroleum ether. The orange crystals were transferred to a 250-ml. Erlenmeyer flask and dissolved in 50 ml. of warm benzene. Five milliliters of the appropriate amine dissolved

in 25 ml. of benzene were added to the benzene solution of the acid chloride with cooling under the tap. When the addition was complete, the mixture was warmed for about an hour on the steam plate. The benzene solution was cooled and washed with water, then extracted with dilute

TABLE II  
TOXICITIES TOWARD HOUSEFLIES OF SOME DERIVATIVES OF  
 $\alpha$ -CYANO- $\beta$ -3,4-METHYLENEDIOXYPHENYLACRYLIC ACID

Formula of compound in which R is	M.p. °C. (uncorr.)	% Nitrogen		G. per 100 ml.	G. pyrethrins per 100 ml.	% Kill	% Knock-down*	O.T.I. % kill
		Calcd.	Found					
								
$\text{R}-\overset{\text{O}}{\parallel}{\text{C}}-\overset{\text{H}}{\text{N}}-\text{C}_6\text{H}_{11}$ (N-Cyclohexyl amide)	177-8	9.44	9.42	0.50 0.50 0.25 0.25 0.125 0.125 0.06 0.06	0.05 0.025 0.05 0.025 0.05 0.025 0.05 0.025	93 92 95 87 88 81 78 60		55 52 55 52 55 51 55 55
$\text{R}-\overset{\text{O}}{\parallel}{\text{C}}-\overset{\text{H}}{\text{N}}-i-\text{C}_6\text{H}_{11}$ (N-iso Amyl amide)	114-5	9.75	9.60	0.5	0.025	74		44
$\text{R}-\overset{\text{O}}{\parallel}{\text{C}}-\text{N} \begin{matrix} \nearrow n-\text{C}_4\text{H}_9 \\ \searrow n-\text{C}_4\text{H}_9 \end{matrix}$ (N-Di-n-butyl amide)	105	8.55	8.77	0.5	0.025	45		43
$\text{R}-\overset{\text{O}}{\parallel}{\text{C}}-\text{N}(\text{CH}_2)_5$ (Piperidide)	134-5	9.85	9.78	0.5 0.5	0.025 —	72 26	53	50 51
Pyrethrins alone				— —	0.05 0.025	36 19	98 93	51 48

\* See footnote in Table I.

hydrochloric acid and finally with aqueous sodium bicarbonate. The benzene was removed on the steam bath and the residue stirred with petroleum ether to effect crystallization when possible. The amides prepared are listed in Table I together with their melting points and the analyses of those which were purified. The nitrogen analyses were by the Kjeldahl method except in the case of the piperidide which was analyzed by the Dumas procedure.

*$\alpha$ -Cyano- $\beta$ -piperonylacrylic acid.* Thirty grams of piperonal and 20 g. of methyl cyanoacetate were shaken and cooled under the tap while 75 ml. of alcohol in which 5 g. of sodium had been dissolved were added in small portions. The mixture was poured into 200 ml. of water, heated to dissolve the sodium salt, and acidified with dilute hydrochloric acid. The acid was filtered and recrystallized from alcohol. The yield of material melting at  $231^{\circ}$  was 28 g. The melting point reported in the literature (1, p. 288) for this acid is  $230^{\circ}$ .

*Amides of  $\alpha$ -cyano- $\beta$ -piperonylacrylic acid.* These amides were prepared in the same manner as used for the preparation of those already described in this paper. They are listed in Table II.

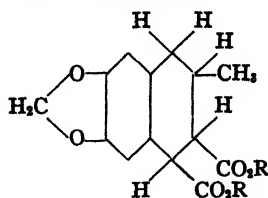
*Esters of maleic acid.* The bis- $\beta$ -chloroethyl, and bis- $\beta$ -bromoethyl esters were made by refluxing maleic anhydride with a 100 per cent excess of the alcohol and 1 g. of *p*-toluene sulfonic acid. An 83 per cent yield of bis- $\beta$ -chloroethyl maleate was obtained. B.p.,  $150$  to  $151^{\circ}/5$  mm.; m.p.  $64.5$  to  $66^{\circ}$ ; anal. calcd. for  $C_8H_{10}O_4Cl_2$ : Cl, 29.4 per cent. Found: (Parr bomb) Cl, 29.0 per cent. The bis- $\beta$ -bromoethyl maleate was obtained in 52 per cent yield after one recrystallization from ethyl alcohol. M.p.  $66.5$  to  $68^{\circ}$ ; anal. calcd. for  $C_8H_{10}O_4Br_2$ : Br, 48.4 per cent. Found: (Parr bomb) Br, 48.4 per cent. The bis- $\beta$ -thiocyanoethyl maleate was obtained in 84 per cent yield from the bromo compound by refluxing 20 g. of the latter with 20 g. of sodium thiocyanate in 50 ml. of alcohol for four hours. The reaction mixture was poured into a large volume of water and extracted with benzene. The residue after removal of the benzene was recrystallized from alcohol. M.p.  $80^{\circ}$ ; calcd. for  $C_{10}H_{10}O_4S_2N_2$ : S, 22.4 per cent. Found: (Parr bomb) S, 22.1 per cent.

*Condensation products between isosafrole and maleic esters.* These were prepared according to the directions of Hudson and Robinson (9) for diethyl maleate except that in the work reported here the products were not distilled but were freed of most of the unchanged starting materials by repeated extraction with petroleum ether. A mixture of 18 g. of isosafrole and 20 g. of ethyl maleate was heated at  $210$  to  $220^{\circ}$  for two hours. The cooled reaction mixture was shaken with five 25-ml. portions of petroleum ether. The petroleum ether was decanted from the oil, the last traces being driven off on a steam bath. The viscous orange-red oil was tested without further purification. Hudson and Robinson reported a 20 per cent yield of the distilled product, b.p.  $205$  to  $210^{\circ}/1.3$  mm. In the case of the tetrahydrofurfuryl maleate-isosafrole condensation, the crude reaction mixture which had not been extracted with petroleum ether was tested against houseflies. The results reported in Table III show that there is no advantage in the purification step, as the crude mixture was shown to be at least as toxic as the partially purified material.



TABLE III

TOXICITIES TOWARD HOUSEFLIES OF THE MALEIC ESTER-ISOSAFROLE CONDENSATION PRODUCTS OF THE TYPE:



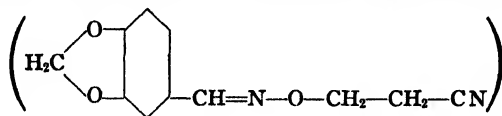
R	G. per 100 ml.	G. pyrethrins per 100 ml.	% Kill	% Knock-down*	O.T.I. % kill
$C_2H_5$ -(Ethyl)	1.00	0.025	99	82	58
	0.50	0.10	95		39
	0.50	0.025	76		41
	0.50	—	61		48
$C_3H_7$ -( <i>n</i> -Propyl)	1.00	0.025	99	93	46
	0.50	0.025	93		46
	0.50	—	55		55
	0.25	0.025	76		46
$C_4H_9$ -( <i>n</i> -Butyl)	0.5	0.10	82	76	39
	0.5	0.025	86		48
	0.5	—	37		55
	0.25	0.10	78		39
	0.25	0.025	74		51
$C_6H_5CH_2$ -(Benzyl)	0.5	0.05	83	65	50
	0.5	—	33		50
	0.25	0.05	71		50
	0.125	0.05	60		50
$C_6H_{11}$ -(Cyclohexyl)	1.00	0.025	92		56
$ClC_2H_4$ -( $\beta$ -Chloroethyl)	0.50	0.025	83		56
$NCSC_2H_4$ -( $\beta$ -Thiocyanoethyl)	1.00	0.025	57	77	50
	0.50	—	4		50
	0.25	0.025	42		50
$C_4H_7OCH_2$ -(Tetrahydrofurfuryl)	0.50	0.10	90	76	39
	0.50	0.025	60		47
	0.50	—	15		48
Tetrahydrofurfuryl (Test made on crude reaction mixture)	0.50	0.025	67	66	48
	0.50	—	51		39
	0.25	0.025	54		39
	0.25	—	32		39
Pyrethrins alone	—	0.10	40	98	39
	—	0.05	36	98	55
	—	0.025	19	93	48

\* See footnote in Table I.

*Piperonaldoximino-β-cyanoethyl ether.* Acrylonitrile (3.5 g.) was added dropwise to a stirred mixture of 10 g. of piperonaldoxime, 30 ml. of dioxan, and 30 ml. of water containing 0.5 g. of sodium hydroxide. The mixture was stirred at room temperature for three hours after which it was poured into 500 ml. of water, neutralized with dilute hydrochloric acid, and extracted with benzene. Removal of the benzene left a residue which, after recrystallization from absolute alcohol-petroleum ether, melted at 65 to 66°. Anal. calcd. for  $C_{11}H_{10}O_3N_2$ : N, 12.8. Found: N, 12.2. The results obtained with this compound are reported in Table IV.

TABLE IV

TOXICITY TOWARD HOUSEFLIES OF PIPERONALDOXIMINO-β-CYANOETHYL ETHER



G. per 100 ml.	G. pyrethrins per 100 ml.	% Kill	% Knockdown*	O.T.I. % kill
1.0	0.05	95		52
1.0	0.025	90		37
1.0	—	76	87	52
0.5	0.05	92		52
0.5	—	28	92	37
0.25	0.05	73		52
0.25	0.025	72		52

\* See footnote in Table I.

"Deo-base" (*purified kerosene fraction*). This odorless petroleum fraction was obtained from S. B. Penick and Company, New York City.

*Official Test Insecticide (O.T.I.) (10)*. This standard consists of a 0.1 per cent solution of pyrethrins in "Deo-base" and was supplied by the National Association of Insecticide and Disinfectant Manufacturers, Inc., New York City.

#### METHOD OF TESTING

The sprays were prepared by dissolving in a small amount of acetone (usually 10 ml.) a quantity of the compound necessary to give the desired concentration when diluted to 100 ml. with "Deo-base." Whenever tests were run with pyrethrins to ensure a high knockdown, these were added to the samples as a kerosene extract.

The samples were tested in a Peet-Grady chamber according to the standard procedure (11). The knockdown values reported in the tables are percentages of the flies rendered unable to fly after a period of ten minutes. The kills are percentages dead after 24 hours. Tests run on the same culture of flies using the Official Test Insecticide gave an indication

of the resistance to pyrethrins of these flies. The O.T.I. kills reported in the last columns of the tables are the averages of two tests, one run at the beginning and the other at the end of a series of eight to ten samples.

In the tables, knockdown values have not been reported for samples containing pyrethrins as these were added to ensure the high knockdowns necessary for successful Peet-Grady tests.

#### SUMMARY

1. The condensation product between piperonal and benzyl cyanide has been tested and found active as a toxicant toward flies when used as a synergistic agent with pyrethrins.

2. Four substituted amides of  $\alpha$ -phenyl- $\beta$ -3,4-methylenedioxyphenylacrylic acid have been prepared. These possess activity as housefly insecticides when used with pyrethrins. The N-cyclohexyl amide and piperidide, the only ones tested without pyrethrins, exhibited a marked paralyzing action of their own.

3. Four substituted amides of  $\alpha$ -cyano- $\beta$ -3,4-methylenedioxyphenylacrylic acid have been synthesized and shown to act as housefly toxicants. When used alone, the piperidide appeared to have a moderate paralyzing action.

4. Of the amides tested, the N-cyclohexyl amides were the most toxic toward flies.

5. Two previously unreported amides of  $\alpha$ -phenyl- $\beta$ -3,4-methylenedioxyphenylacrylic acid and four new amides of  $\alpha$ -cyano- $\beta$ -3,4-methylenedioxyphenylacrylic acid are reported with their melting points and analyses.

6. The condensation products obtained from isosafrole and maleic esters have been found effective against houseflies as synergists with pyrethrins or by their own paralytic action when used alone in "Deo-base."

7. The effectiveness of the crude reaction mixture obtained from tetrahydrofurfuryl maleate and isosafrole has been shown to be at least as great as that of a product that has been partially purified by petroleum ether extraction.

8. Piperonaldoximino- $\beta$ -cyanoethyl ether has been prepared and shown to be toxic toward houseflies.

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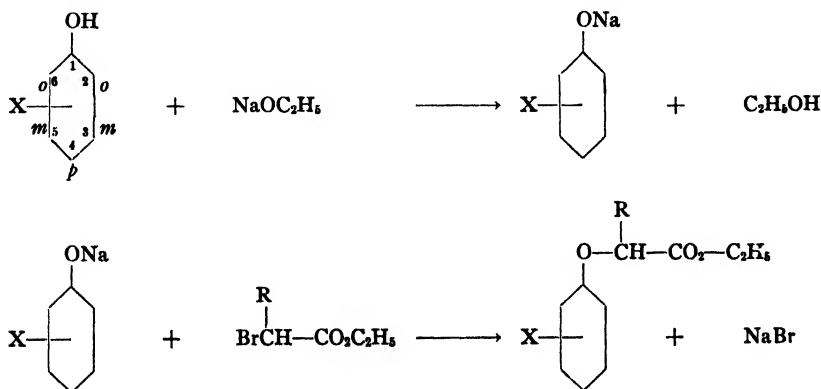


# THE PREPARATION OF SOME SUBSTITUTED PHENOXY ALKYL CARBOXYLIC ACIDS AND THEIR PROPER- TIES AS GROWTH SUBSTANCES

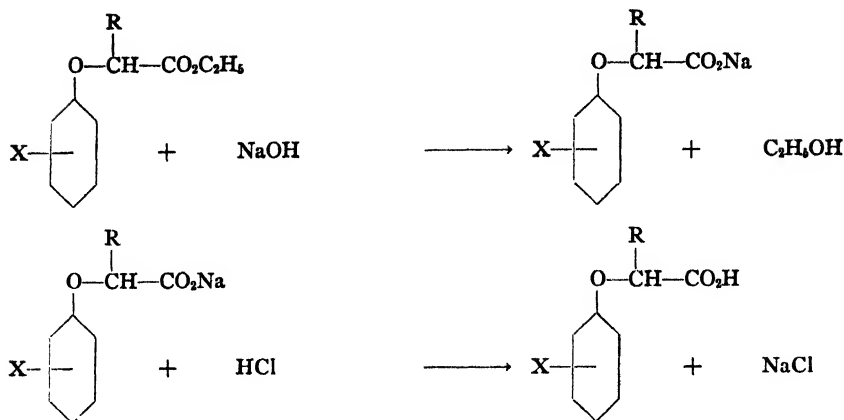
MARTIN E. SYNERHOLM AND P. W. ZIMMERMAN

Within the past few years there has been a rapid rise in the interest shown in the substituted phenoxy acids as plant growth regulating substances. These have been found effective as rooting agents (14), for setting of fruit without pollination (38), for inhibition of bud formation (15), and most recently as herbicides (6, 12, 26, 31). They have been applied in lanolin solutions to localized parts of the plant, as aerosols (38), and as aqueous solutions applied on the plant or to the soil. In consideration of this last method it seemed desirable to have some information regarding the solubilities of these materials in water. The purpose of this paper is to furnish some of this information, to describe new members of this class of compounds, and to present detailed procedures for their preparation.

The phenoxyacetic acids reported in the literature have nearly always been prepared by heating for several hours a water solution of the appropriate phenol, chloroacetic acid, and sodium hydroxide. It has been found that almost quantitative yields of a higher quality product are obtained if the sodium salt of the phenol prepared in absolute alcohol is allowed to react with ethyl bromoacetate for a relatively short time (one hour) followed by saponification of the ester, acidification, and recrystallization of the product. These factors are important where many compounds of high purity are required for greenhouse experiments. Where the ethyl ester of the phenoxy alkyl carboxylic acid is desired, this method permits its recovery by eliminating the saponification step. In this case the solvent is removed and the ester distilled under reduced pressure. The steps in the general procedure are:



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where X may be halogen, alkyl, alkoxy, aryl, nitro, or amino group in one or more of the positions ortho (2, 6), meta (3, 5), or para (4) to the oxygen attached directly to the benzene ring. R is hydrogen (H) in the case of the acetic acid derivatives, methyl (CH<sub>3</sub>) in the propionic, and ethyl (C<sub>2</sub>H<sub>5</sub>) in the butyric acids.

The phenols were in some cases obtained commercially; in others they were prepared from available starting materials. The sources of these are noted in connection with their use in the preparative section of this paper. The ethyl esters of bromoacetic acid,  $\alpha$ -bromopropionic acid, and  $\alpha$ -bromobutyric acid were obtained from Eastman Kodak Company.

#### PREPARATION OF PHENOLS

The trimethyl phenols were prepared by the Clemmensen reduction of the hydroxy dimethyl benzaldehydes. These were obtained in almost quantitative yields by the application of Adams' (1) simplification of the Gattermann aldehyde synthesis.

**Zinc cyanide.** Fifty-nine grams of magnesium chloride hexahydrate in 100 ml. of water and 400 g. of sodium cyanide (95 per cent assay) in 600 ml. of water were mixed and filtered. The filtrate was added to 610 g. of zinc chloride (95 per cent) dissolved in a minimum of 50 per cent ethyl alcohol. The zinc cyanide was filtered, washed with alcohol, then ether, and stored in a desiccator.

**2,6-Dimethyl-4-hydroxybenzaldehyde and 2,4-dimethyl-6-hydroxybenzaldehyde.** Thirty grams of 3,5-xyleneol (0.246 mol.) and 30 g. (0.255 mol.) of zinc cyanide were mixed in 100 ml. of benzene. The mixture was stirred and cooled while dry hydrogen chloride was passed in rapidly for 45 minutes. Aluminum chloride (50 g.) was added in one portion. The mixture was then warmed at 40 to 45° while dry hydrogen chloride was passed in slowly over a period of four and one-half hours. The mixture was poured into 1 liter of 10 per cent hydrochloric acid and steam-distilled until the distillate be-

came clear. The oil which distilled solidified on standing. It was filtered and, after drying, weighed 10 g. and melted at 46 to 48°. This material is 2-hydroxy-4,6-dimethyl benzaldehyde (lit. m.p. 48°). The residue remaining in the flask was cooled and filtered. After drying, it weighed 23 g. and melted at 188° (lit. m.p. 189 to 190° for 2,6-dimethyl-4-hydroxybenzaldehyde).

*2,5-Dimethyl-4-hydroxybenzaldehyde and 2,5-dimethyl-6-hydroxybenzaldehyde.* The procedure differed from the preceding preparation only in the amounts of chemicals used. Thirty-four grams of 2-hydroxy-1,4-dimethyl benzene (Eastman Kodak Co.), 33 g. of zinc cyanide, 110 ml. of benzene, and 57 g. of aluminum chloride were used. The steam distillation yielded 3 g. of oil, which later solidified, melting at 48° (2,5-dimethyl-6-hydroxybenzaldehyde, lit. m.p. 62 to 63°) and 20 g. of non-volatile aldehyde, m.p. 131–132° from water (2,5-dimethyl-4-hydroxybenzaldehyde, lit. m.p. 132°).

*3,4,5-Trimethylphenol.* Mossy zinc (100 g.) was amalgamated by shaking for a few minutes with 10 g. of mercuric chloride, 5 ml. of concentrated hydrochloric acid, and 150 ml. of water. The solution was decanted and the zinc placed in a 1-liter round-bottomed flask with 75 ml. of water, 175 ml. of concentrated hydrochloric acid, and 100 ml. of toluene. Thirty-five grams of 2,6-dimethyl-4-hydroxybenzaldehyde were added and the mixture refluxed for 24 hours, then steam-distilled. The distillate was extracted with benzene. Removal of the benzene left 25 g. (79 per cent) of crystalline product, m.p. 106 to 107° (lit. m.p. 107°).

*2,3,5-Trimethylphenol.* Ten grams of 2,4-dimethyl-6-hydroxybenzaldehyde, 25 g. of amalgamated zinc, 35 ml. of concentrated hydrochloric acid, 15 ml. of water, and 20 ml. of toluene gave by a procedure analogous to the preceding preparation 9 g. of 2,3,5-trimethylphenol, m.p. 93° (lit. 95°).

*2,4,5-Trimethylphenol.* Twenty grams of 2,5-dimethyl-4-hydroxybenzaldehyde, 50 g. of amalgamated zinc, 70 ml. of concentrated hydrochloric acid, 30 ml. of water, and 40 ml. of toluene yielded 16 g. of 2,4,5-trimethylphenol, m.p. 66° (lit. 70 to 71°).

*2,3-Dimethylphenol.* This phenol was prepared according to the procedure of Short, Stromberg, and Wiles (30).

*2,3,4-Trichlorophenol.* The procedure of Hodgson and Kershaw (16) was followed.

*o-Cresol (o-methylphenol).* *o*-Toluidine (Eastman Kodak Co.) (100 g.) was dissolved in 400 ml. of water and 200 ml. of concentrated sulphuric acid, and cooled to 0 to 5° in an ice-salt mixture. A solution of 65 g. of sodium nitrite in 100 ml. of water was added slowly to the stirred and cooled mixture. After the addition, the mixture was warmed at 40 to 50° and finally steam-distilled until the distillate became clear. The distillate was extracted with benzene and distilled (b.p. 114 to 115°/63 mm.).



*m-Cresol.* Starting with *m*-toluidine, the same procedure was followed as for the ortho isomer. The product boiled at 122 to 123°/60 mm.

*p-Cresol.* *p*-Toluidine gave, with the same procedure as for the ortho and meta isomers, the *p*-cresol boiling at 103°/23 mm.

*2-Methyl-4-bromophenol.* 2-Methyl-4-bromoaniline (Eastman Kodak Co.) (10 g.) was dissolved in 50 ml. of hydrochloric acid and 300 ml. of water by heating to boiling. The mixture was cooled to 0 to 5° and diazotized with 3.7 g. of sodium nitrite. The mixture was then warmed at 40 to 50° until evolution of nitrogen had practically ceased, then steam-distilled; yield: 7.5 g., m.p. 65°.

*2,4-Dimethyl-6-bromophenol (9).* 2,4-Dimethyl aniline (Eastman Kodak Co.) (25 g.) in 100 ml. of glacial acetic acid was treated with 33 g. of bromine in 100 ml. of acetic acid. The bromine solution was added dropwise to the stirred and ice-cooled mixture. After the addition, the mixture was poured into 500 ml. of water, filtered by gravity to remove tar, boiled with "Norit," and made alkaline. The amine was filtered, diazotized, and steam-distilled.

*2-Bromo-4-methylphenol (8).* 2-Bromo-4-methylaniline (Eastman Kodak Co.) was diazotized in 18 to 20 per cent hydrochloric acid and steam-distilled. The oil obtained was not purified beyond the steam distillation.

*2,6-Dibromo-4-methylphenol.* 2-Bromo-4-methylaniline (Eastman Kodak Co.) was brominated in glacial acetic acid by the procedure for the bromination of 2,4-dimethyl aniline, using 32 g. of the amine in 100 ml. of glacial acetic acid and 27.5 g. of bromine in the same amount of that solvent. The dibromo toluidine formed (39 g.) was diazotized in 200 ml. of water and 50 ml. of concentrated sulphuric acid with 10.5 g. of sodium nitrite in 25 ml. of water, then steam-distilled.

*2-Methoxy-4-methylphenol (creosol) (10).* This material was obtained by the reduction of vanillin (45 g.), using amalgamated zinc (100 g.), hydrochloric acid (175 ml.), water (75 ml.), and toluene (100 ml.). The procedure was the same as that used in the preparation of the trimethylphenols.

*3,4-Dichlorophenol.* 3,4-Dichloronitrobenzene was reduced catalytically in 10 g. portions in 100 ml. of alcohol with 0.1 g. of platinum oxide catalyst in an Adams shaker. The 3,4-dichloroaniline so formed was diazotized and steam-distilled in the usual manner.

*2,5-Dichlorophenol (25).* 2,5-Dichloroaniline was diazotized and steam-distilled in the usual way.

*2-Chloro-4-methylphenol (28).* *p*-Cresol was treated with an equivalent amount of sulphuryl chloride.

*4-Chloro-2-methylphenol (28).* *o*-Cresol was chlorinated by the use of one equivalent of sulphuryl chloride.

*4-Chloro-3,5-dimethylphenol (21).* Chlorination of 1,3,5-xylene in

chloroform, using one equivalent of sulphuryl chloride, gave the desired 4-chloro derivative.

*2-Chloro-3,5-dimethylphenol* (21). This material resulted as a by-product in the preparation of the 4-chloro xlenol.

*2,4-Dichloro-3,5-dimethylphenol* (21). The action of two equivalents of sulphuryl chloride on 1,3,5-xlenol yielded the dichloro derivative.

The following phenols were obtained from Eastman Kodak Company: *o*-chloro-, *m*-chloro-, *p*-chloro-, 2,4-dichloro-, 2,4,5-trichloro-, 2,4,6-trichloro-, 4-chloro-3-methyl-, 3,4-dimethyl-, 3,5-dimethyl-, *o*-nitro-, *p*-nitro-, *p*-fluoro-, methyl salicylate (*o*-carbomethoxyphenol), *p*-chlorothymol, *p*-thiocresol, and 2,4-dichloro- $\alpha$ -naphthol.

*p*-Phenylphenol. This material was obtained from Dow Chemical Company.

*4,6-Di-tert butyl-m-cresol*. This material was kindly supplied by Koppers Company (Tar and Chemical Division).

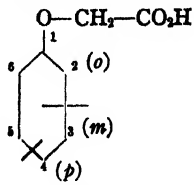
#### PREPARATION OF THE PHENOXY ALKYL CARBOXYLIC ACIDS

The members of the acetic, propionic, and butyric acid series listed in Tables I, II, and III were all prepared in the same general manner using ethyl bromoacetate, ethyl  $\alpha$ -bromopropionate, or ethyl  $\alpha$ -bromobutyrate and the sodium salt of the substituted phenol, followed by saponification and purification by crystallization. Details are given here for the preparation of 2,4-dichlorophenoxyacetic acid.

*2,4-Dichlorophenoxyacetic acid*. Three and six-tenths grams (0.156 atom) of sodium were dissolved in 100 ml. of absolute alcohol and 25 g. (0.153 mol.) of 2,4-dichlorophenol were added when the sodium had disappeared. To the sodium dichlorophenoxide solution so prepared were added 26 g. (0.155 mol.) of ethyl bromoacetate. Sodium bromide began to precipitate immediately. The mixture was allowed to stand on a steam plate at incipient boiling for one hour after which 100 ml. of 10 per cent aqueous sodium hydroxide were added. The mixture was again warmed for a few minutes, then diluted with 500 ml. of water. A few grams of decolorizing carbon ("Norit-A") were added after which the mixture was boiled for a few minutes, then filtered through a Büchner funnel which had previously been heated to prevent crystallization in the holes of the funnel. The filtrate was acidified with dilute hydrochloric acid and cooled to 0°. The crude product, when air-dried, weighed 34 g. and melted at 133 to 137°. It was purified by dissolving in a minimum amount of boiling benzene, cooling, and adding an equal volume of petroleum ether. After standing overnight at 0°, the white crystalline 2,4-dichlorophenoxyacetic acid was filtered and washed with a little petroleum ether. The yield was 32 g. (95 per cent) of product melting at 139 to 140° (uncorr.).

*Bis-(o-chlorophenoxy)acetic acid*. Thirty grams of chlorophenol, 15 g. of

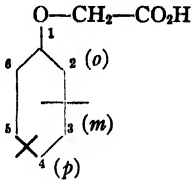
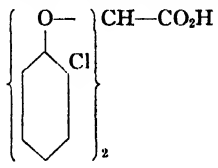
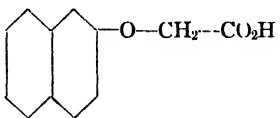
TABLE I  
SUBSTITUTED PHENOXYACETIC ACIDS

	M.p. °C. uncorr.*	Neutral equivalent		Solubility (20° ± 1° C.) in water, mg./liter	
		Calcd.	Found		
<i>o</i> -Chloro- (3, 13, 19, 22, 31, 34, 37)	144-6 (143-5)			1120	1127
<i>m</i> -Chloro- (13, 19)	110 (108-10)			1293	1178
<i>p</i> -Chloro- (19, 22, 31, 34, 35, 37)	157-8 (156-7)			681	674
2,4-Dichloro- (2, 6, 7, 12, 23, 26, 27, 31, 34, 35, 37)	139-40 (140)			546	540
2,5-Dichloro-	142-3	221	222		
3,4-Dichloro-	141	221	222	530	520
2,4,5-Trichloro- (12, 27, 35)	154-5 (157-8)			194	189
2,4,6-Trichloro- (31, 35)	190			154	148
2,3,4-Trichloro-	150-60	255.5	257		
<i>o</i> -Methyl- (13, 19, 31)	153 (151-2)			490	495
<i>p</i> -Methyl- (13, 19, 31)	136-7 (134-6)			1200	1203
3,5-Dimethyl- (29, 39)	110-1 (109)			804	800
3,4,5-Trimethyl- (20)	149 (149)			233	231
2,4,5-Trimethyl-	129	194.1	193	268	272
2,3,5-Trimethyl-	125	194.1	194	531	547
4-Chloro-2-methyl- (31)	119-20	200.5	201		
2-Chloro-4-methyl-	138-9	200.5	200		
4-Chloro-3-methyl- (31)	177-8	200.5	203		
4-Chloro-3,5-dimethyl-	151	214.5	213		
2-Chloro-3,5-dimethyl-	140-2	214.5	215		
2,4-Dichloro-3,5-dimethyl-	146-7	249	247		
4-Bromo-2-methyl-	118	245	241		
6-Bromo-2,4-dimethyl-	152	259	260	197	192
2-Bromo-4-methyl-	145	245	245		
2,6-Dibromo-4-methyl-	200	324	323		

sodium hydroxide, and 12.9 g. of dichloroacetic acid were mixed in 100 ml. of water. The mixture was heated overnight on a steam plate, poured into a large volume of water, acidified, and filtered. The crude acid was recrystallized from benzene. The pink crystals when pure melted at 142°.

*Neutral equivalents.* The neutral equivalents reported in the table for the new compounds are analytical figures indicating the identity and purity of the material. They were obtained by titration of an alcoholic solution of the acid against standard 0.1 N sodium hydroxide and are expressed as the weight of the acid necessary to exactly neutralize one equivalent (40 g.) of sodium hydroxide.

TABLE I (Continued)

	M.p. °C. uncorr.*	Neutral equivalent		Solubility (20° ± 1° C.) in water, mg./liter
		Calcd.	Found	
<i>p</i> -Phenyl	189-90	228	228	1895 1900
<i>o</i> -Nitro- (13)	156-7 (158)			
<i>o</i> -Carboxy- (-CO <sub>2</sub> H)	187-8	98	98	
2,4-Di- <i>tert</i> butyl-5-methyl-	153	278.4	279	
2- <i>iso</i> -Propyl-4-chloro-5-methyl-	138	242.6	244	
	142	314	313	463 461
[Bis ( <i>o</i> -chlorophenoxy) acetic acid]				
	155 (154)			283 279
( $\beta$ -Naphthoxyacetic acid) (2, 4, 5, 7, 11, 17, 18, 19, 24, 31, 33, 34, 36)				

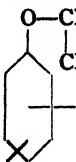
\* The figures in parentheses in column 2 are melting points previously recorded in the literature. Note: Compounds not followed by reference numbers are reported for the first time.

#### METHOD OF DETERMINING THE SOLUBILITIES

The solubilities reported in the tables are the amounts of the substances remaining in solution after allowing a warm saturated solution to cool to 20° ± 1° C. It was felt that the method would give sufficiently accurate data for the work in which they are to be used. The determinations were run in duplicate.

About 5 g. of the pure phenoxy compound were warmed to about 80° with approximately 110 ml. of distilled water, then stoppered and set aside for 5 days in a room the temperature of which remained at 20° ± 1° C. The contents of the flask were filtered into a 100 ml. volumetric flask, this manipulation being carried out in the 20° room. Exactly 100 ml. of the filtrate were titrated with standard alkali using phenolphthalein and, from the formula, the weight in milligrams of dissolved phenoxy compound was computed. This figure multiplied by ten gave the solubility of the com-

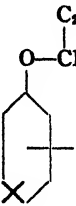
TABLE II  
SUBSTITUTED  $\alpha$ -PHENOXYPROPIONIC ACIDS

	M.p. °C. uncorr.*	Neutral equivalent		Solubility (20° ± 1° C.) in water, mg./liter	
		Calcd.	Found		
<i>o</i> -Chloro- (35)	114-5			1575	1572
<i>m</i> -Chloro-	113-4	200.5	202	912	900
2,4-Dichloro- (35)	117-8 (117-8)			500	495
2,4,5-Trichloro- (35)	177-8 (177-8)			54	54
2,4,6-Trichloro-	115-6				
<i>o</i> -Methyl-	93-4	180.2	181		
<i>m</i> -Methyl-	100-2				
<i>p</i> -Methyl-	101-2	180.2	182	2260	2270
3,4,5-Trimethyl-	115-6	208.1	208	499	492
4-Chloro-2-methyl-	92-3	214.5	214		
2-Chloro-4-methyl-	117	214	214		
4-Chloro-3-methyl-	90-1	214	213		
4-Chloro-3,5-dimethyl-	137-8	228.5	227		
2,4-Dichloro-3,5-dimethyl-	148-9	263	261		
$\beta$ -Naphthoxypropionic acid (2, 17, 18)	107			398	402

\* See footnote in Table I.

pound in milligrams per liter at 20° ± 1° C. The solubilities are given in duplicate in Tables I, II, and III.


TABLE III  
SUBSTITUTED  $\alpha$ -PHENOXYBUTYRIC ACIDS

	M.p. °C. uncorr.	Neutral equivalent		Solubility (20° ± 1° C.) in water, mg./liter	
		Calcd.	Found		
<i>m</i> -Chloro-	74-4.5	214.5	214		
<i>o</i> -Methyl-	50	194.2	196		
<i>m</i> -Methyl-	73	194.2	194		
<i>p</i> -Methyl-	62-3	194.1	192	2895	2900
3,4,5-Trimethyl-	70-2	222	222		
2,4,5-Trimethyl-	86-7	222	222	186	186

## TESTING FOR PHYSIOLOGICAL ACTIVITY

A growth substance may induce several different responses in the plant. These are cell elongation (resulting in curvature), adventitious root formation, cell division, parthenocarp (fruit set without pollination, resulting in seedless or partially seedless fruit), and modification of organs. To these

TABLE IV  
THE PHYSIOLOGICAL PROPERTIES OF SOME SUBSTITUTED PHENOXYACETIC ACIDS

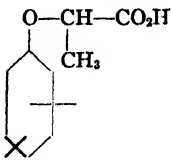
	Activity for cell elongation, threshold concentration mg./g. lanolin	Activity for modification, threshold concentration mg./g. lanolin
3,4-Dichloro- 2,6-Dichloro- (32)* 2,5-Dichloro- (31) 2,4-Dichloro- 2,3,4-Trichloro- <i>m</i> -Methyl- (13, 19, 31) 2,3-Dimethyl- (29) 3,4,5-Trimethyl- 2,4,5-Trimethyl- 2,3,5-Trimethyl- <i>p</i> -Phenyl- <i>o</i> -Nitro- <i>o</i> -Amino- 4-Chloro-2-methyl- (31) 2-Chloro-4-methyl- 4-Chloro-3-methyl- (31) 4-Chloro-3,5-dimethyl- 2-Chloro-3,5-dimethyl- 2,4-Dichloro-3,5-dimethyl- 4-Bromo-2-methyl- 6-Bromo-2,4-dimethyl- 2-Bromo-4-methyl- 2,6-Dibromo-4-methyl- <i>o</i> -Carboxy- 2,4-Di- <i>tert</i> butyl-5-methyl- 2- <i>iso</i> -Propyl-4-chloro-5-methyl- 2-Methoxy-4-methyl- (10)	0.1 Inactive 0.25 0.05 1.0 5.0 0.5 1.0 0.25 0.5 Inactive Inactive Inactive 0.1 1 0.5 1 0.1 1 0.05 Inactive 5 Inactive Inactive Inactive Inactive Inactive Inactive	Inactive Inactive Inactive 0.05 0.05 Inactive 5.0 0.1 0.1 0.5 Inactive Inactive Inactive Inactive Inactive 0.1 1.0 1.0 0.1 0.05 Inactive 10 Inactive Inactive Inactive Inactive Inactive
<i>Bis</i> ( <i>o</i> -chlorophenoxy) acetic acid	Inactive	10

\* Obtained from Dr. D. S. Tarbell, University of Rochester, Rochester, N. Y.

effects may be added the herbicidal action due to high concentrations of the growth substance. This last should be distinguished from the ordinary toxicity or burning action toward plants of many chemicals which show no other physiological response. A chemical inducing one or more of these responses in addition to any possible phytotoxicity is considered a growth substance.

Physiological activity of substances or extracts of plants can be determined by the use of *Avena* coleoptiles or actively growing green plants. A simple method using young tomato (*Lycopersicon esculentum* Mill.) plants in light or dark has been perfected in this laboratory. It has an advantage over the *Avena* coleoptile because experiments can be conducted in light or dark. Also the coleoptile method detects only cell elongation whereas with one treatment the tomato plant can detect the capacity of a chemical to induce cell elongation, cell division, adventitious roots, and formative effects. The time required varies with the response. Detection of cell

TABLE V  
THE PHYSIOLOGICAL PROPERTIES OF SOME SUBSTITUTED  $\alpha$ -PHENOXYPROPIONIC ACIDS

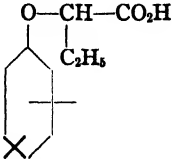
	Activity for cell elongation, threshold concentration mg./g. lanolin	Activity for modification, threshold concentration mg./g. lanolin
<i>m</i> -Chloro-	0.25	Inactive
2,4,6-Trichloro-	20	20
<i>o</i> -Methyl-	0.1	Inactive
<i>m</i> -Methyl-	0.5	1.0
<i>p</i> -Methyl-	1.0	0.5
3,4,5-Trimethyl-	1.0	0.1
2,4-Dichloro-	0.1	0.003
4-Chloro-2-methyl-	0.01	Inactive
2-Chloro-4-methyl-	1.0	1.0
4-Chloro-3-methyl-	1.0	Inactive
4-Chloro-3,5-dimethyl-	1.0	0.1
2,4-Dichloro-3,5-dimethyl-	0.1	0.1

elongation requires only 20 minutes to two hours, cell division 48 to 72 hours, initiation of roots five to ten days, and formative effects four to ten days. If the chemical does not induce at least one of these responses, it is considered inactive.

A simple method for testing new chemicals involves the following procedure: a lanolin solution is prepared by mixing 10 to 20 mg. of the substance with one gram of melted lanolin. When these are thoroughly mixed, a small amount of the preparation is applied with a glass rod to the upper side of a young tomato leaf and of the adjacent stem on the side facing the treated leaf. The angle between the stem and the leaf before treatment is usually near 45 degrees. If the chemical is active, causing cell elongation, the leaf moves downward and the stem curves away from the treated side, thus increasing the angle. The same plant is kept for 10 to 12 days to determine the effect on cell division, root-inducing activity, and formative effects. If activity is indicated by the first test, the chemicals are then

studied in comparison with a standard such as  $\alpha$ -naphthaleneacetic acid which is active when used at 0.001 per cent in lanolin (0.01 mg./g. of lanolin). The lowest concentration which would induce responses (threshold concentration) is reported for each chemical (Table IV, V, or VI).

TABLE VI  
THE PHYSIOLOGICAL PROPERTIES OF SOME SUBSTITUTED  $\alpha$ -PHENOXYBUTYRIC ACIDS

	Activity for cell elongation, threshold concentration mg./g. lanolin	Activity for modification, threshold concentration mg./g. lanolin
<i>m</i> -Chloro-	0.5	Inactive
<i>o</i> -Methyl-	0.05	Inactive
<i>m</i> -Methyl-	0.5	0.5
<i>p</i> -Methyl-	20	5.0
3,4,5-Trimethyl-	1.0	Inactive
2,4,5-Trimethyl-	0.5	0.5

Comparisons can also be made by applying the chemical to the soil of the potted plant. For example, 1 to 10 mg. of an active chemical in 50 cc. of water applied to the soil of a potted plant will cause the entire plant to show an epinastic response. This then is usually followed by the three other responses described above.

#### SUMMARY

1. Detailed, generally applicable, directions are given for the preparation of substituted phenoxy alkyl carboxylic acids.
2. Several previously unreported substituted phenoxyacetic, propionic, and butyric acids are reported with their melting points and their effects as plant growth substances.
3. Solubilities in water at  $20^{\circ} \pm 1^{\circ}$  C. are given with the method of obtaining these for several substituted phenoxy alkyl carboxylic acids.
4. Detailed directions are given for testing the compounds as plant growth substances.
5. The results found in the present investigation indicate that halogen atoms or methyl groups are of most importance in lending activity for cell enlargement to the phenoxy alkyl carboxylic acids. The halogens are generally more effective than methyl groups.
6. The 2, 3, and 4 positions in the benzene ring are those in which the substituents exert their greatest influence.



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# OUTSTANDING DISEASES OF AGRICULTURAL CROPS AND USES OF FUNGICIDES IN THE UNITED STATES<sup>1</sup>

S. E. A. McCALLAN<sup>2</sup>

It is generally stated that plant diseases in the United States cause an estimated annual loss of about two billion dollars. This loss is the resultant of many thousand different plant diseases of varying importance, not only inherently, but also from season to season and from locality to locality. One of the most widely used and effective methods of combating these diseases is, of course, by the use of fungicides. In the course of developing laboratory and greenhouse methods of evaluating new chemicals as potential fungicides, the specificity of many of the materials, especially the organic chemicals, is increasingly evident. It thus becomes essential to decide what organisms and diseases shall be selected for test, since obviously only a few may be chosen. Therefore, we are faced with the question as to what are the most important diseases of agricultural crops in the United States, also how effective are the present fungicides and what are their main uses. These answers involve many very complex problems which for the most part no attempt has been made to solve; however, some points of view are here presented in the hopes that others may be stimulated to provide a better answer.

## OUTSTANDING DISEASES

The economic importance of a plant disease as understood here refers to its importance after the customary control measures have been taken and not to its potential destructiveness if no control were attempted. Two primary considerations determine the economic importance of a plant disease—first, the value of the crop and secondly, the extent of the losses. Usually the determination of loss has not proceeded beyond this point, the estimated monetary loss being calculated from the per cent disease loss. Some of the pitfalls have been pointed out even for this simple estimate by Stevens (10), Hyslop (5), and Smith *et al.* (9). For example, if the disease had been controlled, more crop would have been produced and the value probably changed; also, one or more diseases or insect pests may affect the same diseased plant part at the same time and the losses are thus not additive.

The 50 leading agricultural crops of the United States are given in

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TABLE I  
MAJOR AGRICULTURAL CROPS OF UNITED STATES

Crop	1937-1941	1930-1939	Farm value per acre \$	Leading states and % of total production 1930-39				
	Farm value \$1,000,000	Acreage 1,000,000		1st	2nd	3rd	4th	5th
1. Corn	1,502	98.0	15	Iowa 17	Ill. 14	Ind. 7	Minn. 6	Ohio 6
2. Cotton	813	31.2	26	Texas 28	Miss. 12	Ark. 10	Ala. 9	Ga. 9
3. Wheat	669	55.9	12	Kan. 18	N. D. 9	Okla. 7	Wash. 6	Neb. 6
4. Hay, Tame	661	56.1	12	N. Y. 7	Wisc. 7	Cal. 6	Iowa 6	Minn. 5
5. Oats	345	36.5	10	Iowa 19	Minn. 13	Ill. 12	Wisc. 8	Ohio 4
6. Tobacco	288	1.68	171	N. C. 38	Ky. 23	Tenn. 8	Va. 7	S. C. 6
7. Potatoes	225	3.30	68	Maine 12	N. Y. 8	Mich. 7	Idaho 7	Pa. 7
8. Barley	125	10.7	12	Minn. 20	Cal. 13	N. D. 11	S. D. 10	Wisc. 10
9. Soybeans*	100†	2.1	48	Ill. 48	Iowa 15	Ind. 14	Ohio 12	
10. Oranges	97‡			Cal. 62	Fla. 35	Texas 2		
11. Apples	95			Wash. 23	N. Y. 14	Va. 8	Pa. 7	Cal. 6
12. Beans, Dry Snap	73 51 22	1.9	27	Mich. 31	Cal. 30	Idaho 11	Colo. 9	N. Y. 8
13. Tomatoes	59	0.25	88	Fla. 27	Cal. 8	N. J. 7	Md. 7	N. Y. 7
14. Sugar beets	56	0.56	105	Cal. 18	Ind. 16	N. J. 10	Md. 9	N. Y. 7
15. Sweet potatoes	56	0.82	68	Colo. 23	Cal. 18	Neb. 9	Mich. 9	Mont. 8
16. Sorghums	56	0.88	64	Ga. 12	N. C. 11	Ala. 11	Miss. 10	La. 9
17. Peaches	52	10.9	5	Texas 53	Okla. 14	Kan. 14	N. M. 4	Cal. 4
18. Peanuts	51			Cal. 42	Ga. 9	N. C. 4	Ark. 3	Pa. 3
19. Grapes	50	1.5	34	Ga. 31	N. C. 23	Ala. 15	Va. 14	Texas 8
20. Hay, Wild	48			Cal. 88	N. Y. 3	Mich. 2	Ohio 1	Pa. 1
21. Rice	43	11.8	4	Neb. 17	Minn. 16	N. D. 15	S. D. 10	Kan. 6
22. Flaxseed	42‡	0.94	46	La. 41	Texas 23	Ark. 18	Cal. 18	
23. Strawberries	33	1.8	23	Minn. 53	N. D. 26	S. D. 7	Cal. 7	Mont. 4
24. Lettuce	33	0.18	183	La. 11	Cal. 8	Ore. 7	Ark. 7	Tenn. 7
25. Peas, Dry	31 7	0.16	206	Cal. 65	Ariz. 17	N. Y. 5	Wash. 4	Colo. 3
Green	24	0.26	27	Wash. 42	Idaho 32	Mont. 9	Colo. 8	Wisc. 4
26. Sugar cane	27	0.37	65	Wisc. 28	N. Y. 10	Cal. 9	Wash. 7	Utah 6
27. Rye	22	0.39	69	La. 89	Fla. 11			
28. Pears	22	0.39	7	N. D. 20	Minn. 17	S. D. 12	Neb. 8	Wisc. 7
29. Onions	20	3.32		Cal. 36	Wash. 19	Ore. 12	N. Y. 5	Mich. 4
30. Celery	20	0.13	160	N. Y. 18	Texas 16	Mich. 13	Cal. 8	Colo. 8
31. Lemons	19	0.036	556	Cal. 31	Fla. 20	N. Y. 17	Mich. 15	N. J. 4
32. Grapefruit	18			Cal. 100				
33. Prunes, plums	17			Fla. 61	Texas 26	Cal. 7	Ariz. 6	
34. Cabbage	16			Cal. 79	Ore. 14	Wash. 4	Idaho 2	Mich. 1
35. Cantaloupes	15	0.175	91	N. Y. 20	Texas 9	Wisc. 8	Pa. 4	Colo. 3
36. Asparagus	14	0.121	124	Cal. 48	Ariz. 10	Colo. 9	Md. 5	Mich. 4
37. Cherry	13	0.110	127	Cal. 67	N. J. 17	Wash. 4	S. C. 4	Md. 3
38. Alfalfa seed	13			Mich. 22	Cal. 17	N. Y. 15	Wash. 13	Ore. 11
39. Red clover seed	13	0.56	23	Kan. 12	Ariz. 10	Idaho 10	Okla. 9	Neb. 7
40. Velvet beans	13	0.95	14	Ind. 15	Ohio 13	Ill. 12	Mich. 12	Idaho 10
41. Cowpeas, peas	12	2.0	6	Ga. 55	Ala. 24	Fla. 7	S. C. 6	Miss. 5
42. Walnuts	12	1.1	11	S. C. 15	Ala. 14	Texas 13	Ga. 13	Miss. 9
43. Sweet corn†	10			Cal. 95	Ore. 5			
44. Carrots	10	0.34	29	Ill. 20	Tenn. 19	Iowa 11	Ind. 10	Md. 9
45. Apricots	9	0.04	260	Cal. 60	Wash. 11	N. Y. 7	Mich. 3	N. J. 3
46. Hops	9			Cal. 97	Texas 3			
47. Cucumber	9	0.030	287	Ore. 53	Cal. 25	Wash. 22		
48. Watermelon	8	0.13	68	Mich. 14	Cal. 8	Wisc. 6	N. C. 6	N. Y. 6
49. Cranberries	7	0.26	31	Ga. 17	Cal. 17	Texas 10	Fla. 10	S. C. 7
50. Pecans	6	0.028	257	Mass. 68	N. J. 18	Wisc. 11	Wash. 2	Ore. 1
				Texas 37	Okla. 19	Ga. 12	Miss. 8	La. 7

\* As beans.

† For processing only.

‡ 1939-41.

Table I as obtained from the 1937 to 1941 average farm values reported in "Agricultural statistics" (13). If the above direct method of estimating importance is followed, it will be seen that a 0.5 per cent loss in the \$1,500,000,000 corn crop would equal in importance a 50 per cent loss on a \$15,000,000 crop such as cantaloupes or the complete loss of the \$7,000,000 cranberry crop; this seemingly is unreasonable. It would appear that a very small loss on even a major crop can not be considered of major significance, furthermore the growers themselves could not be interested in preventing a very small loss. Since our considerations are to be on a national basis unrestricted by state boundaries or crop divisions, there likewise becomes an eventual limit at the other end and the complete destruction of an exceedingly minor crop could not be considered a major disease even though it was disastrous for the growers concerned.

From these general considerations it appears that the national importance of a plant disease is based on a sliding scale within certain limits. Thus, no disease causing an average of 1 per cent or less of loss, and no crop of \$100,000 or less in value has been considered. After trying various methods a logarithmic scale was finally used so that the product of the logarithm of the per cent disease and the logarithm of the farm value expressed in \$100,000 units gave an index of importance. For example, apple scab causing an average of 6.8 per cent loss of the \$95,000,000 apple crop (i.e., 950 units) would have an index of  $0.83 \times 2.98$  or 2.5. Diseases which by this method would be given an equal rating, say an index of 2.0, are those causing a 3 per cent loss on a \$1,000,000,000 crop, 4 per cent on \$200,000,000, 10 per cent on \$10,000,000, 35 per cent on \$2,000,000, and 100 per cent on \$1,000,000. In the Plant Disease Reporter Supplements (14), more or less complete estimates may be obtained for the annual losses due to the commoner diseases of 21 different crops, the majority of which are among the most important crops. The average losses for the ten-year period, 1930-1939, were thus obtained and those diseases with indices of 1 or greater are recorded in Table II as outstanding diseases.

*Limitations.* In examining Table II, various limitations must be emphasized. The Plant Disease Reporter figures are only estimates, but limited independent sources of estimate have shown reasonably good agreement (12), thus increasing the confidence in the former. However, even were the disease estimates precise and the method of evaluating importance known to follow a natural law, large differences between the indices would be required for significance because of yearly fluctuations in disease. In fact, no distinction has been made between diseases present every year in more or less constant amount and those which are epiphytotic such as stem rust of wheat and late blight of potato. Stevens and Wood (11, 12) consider fluctuations in loss and the resulting economic disturbance more important than total loss. It has not been possible to include this significant

TABLE II  
OUTSTANDING DISEASES OF AGRICULTURAL CROPS IN UNITED STATES

Index	Host	Disease	Organism	Per cent loss 1930-39		Present major control measure
				Mean	Range	
4.0	Corn	Root, stalk & ear rots	<i>Diplodia zeae</i> , <i>Gibberella zeae</i> , <i>Fusarium moniliforme</i> , etc.	9.0	3.8-16.1	General;* organic mercury seed treatment
2.7	Cotton	Anthraxnose, seedling blights & boll rot	<i>Glomerella gossypii</i> , <i>Fusarium</i> spp., <i>Rhizoctonia solani</i> , <i>Sclerotium bataticola</i> , <i>Diplodia gossypina</i> , etc.	5.1	0.2-11.6	Organic mercury seed treatment
2.5	Apple	Scab	<i>Venturia inaequalis</i>	6.8	2.8-14.1	Sulphur & copper sprays & dusts
2.1	Cotton	Wilt	<i>Fusarium vasinfectum</i>	3.5	2.2-4.8	Resistant varieties
2.1	Cotton	Root rot	<i>Phymatotrichum omnivorum</i>	3.4	2.3-5.0†	Rotation
2.0	Corn	Smut	<i>Ustilago zeae</i>	3.1	2.6-4.6	General
2.0	Wheat	Stem rust	<i>Puccinia graminis</i>	3.7	tr.-23.0	Resistant varieties; barberry eradication
2.0	Potato	Tip & hopper burn	Physiological and insect	4.1	2.9-6.4	Bordeaux mixture
1.9	Oat	Smut	<i>Ustilago avenae</i> , <i>U. levis</i>	3.7	2.5-4.7	Organic mercury; formaldehyde
1.9	Sweet potato	Stem rot	<i>Fusarium batatis</i> , <i>F. hyperoxysporum</i>	5.1	2.6-12.1	General
1.8	Wheat	Leaf rust	<i>Puccinia triticae</i>	2.9	1.0-9.6	Resistant varieties
1.8	Peach & cherry	Brown rot	<i>Sclerotinia fructicola</i>	4.6	1.8-9.5	Sulphur sprays & dusts
1.8	Pear	Blight	<i>Erwinia amylovora</i>	6.0	1.6-13.6	General
1.7	Oat	Crown rust	<i>Puccinia coronata</i>	3.1	0.3-11.9	Resistant varieties
1.7	Tomato	Early blight	<i>Alternaria solani</i>	4.4	0.7-6.8	Copper sprays & dusts
1.7	Bean	Bacterial blights	<i>Phytophthora phaseoli</i> and var. <i>medicaginis</i>	4.1	1.2-7.9	General
1.7	Pea	Root & stem rots	<i>Aphanomyces</i> spp., <i>Fusarium</i> spp., etc.	5.0	2.1-7.0†	Seed treatments

TABLE II (Continued)

Index	Host	Disease	Organism	Per cent loss 1930-39		Present major control measure
				Mean	Range	
1.6	Oat	Blast	Physiological	2.9	2.5-3.6†	None
1.6	Tobacco	Blue mold	<i>Peronospora tabacina</i>	3.1	1.8-5.0†	Paradichlorobenzene fumigation of seed bed
1.6	Strawberry	Leaf spot	<i>Mycosphaerella fragariae</i>	4.6	2.3-9.0	General; copper sprays & dusts
1.5	Cotton	Bacterial blight	<i>Phytophthora malvacearum</i>	2.5	0.6-7.6	Seed treatment; rotation
1.5	Tobacco	Wild fire	<i>Phytophthora tabacum</i>	2.8	1.3-6.7†	General
1.5	Tobacco	Deficiency	Physiological	2.8	1.7-3.4	Fertilize
1.5	Potato	Late blight	<i>Phytophthora infestans</i>	2.8	0.8-12.8	Bordeaux mixture
1.5	Sugar beet	Leaf spot	<i>Cercospora beticola</i>	3.5	1.5-6.8†	General; copper sprays; seed treatments
1.5	Sweet potato	Black rot	<i>Ceratostomella fimbriata</i>	3.7	1.7-8.6	General; mercury tuber dip
1.4	Cherry	Leaf spot	<i>Coccomyces hiemalis</i>	5.1	0.4-11.9	Sulphur & copper sprays
1.3	Tobacco	Mosaic	Virus	2.5	2.1-2.7†	General
1.3	Potato	Rhizoctoniosis	<i>Rhizoctonia solani</i>	2.4	1.5-3.5	Seed treatments
1.3	Strawberry	Root rots	<i>Sphaeropsis</i> sp., etc.	3.4	1.1-6.4	General
1.2	Potato	Leaf roll	Virus	2.3	1.2-3.5	Certified seed
1.1	Sweet corn	Smut	<i>Ustilago zeae</i>	3.4	1.6-5.0	General
1.0	Potato	Mosaic	Virus	2.0	1.4-2.6	Certified seed
1.0	Sweet corn	Bacterial wilt	<i>Phytophthora stewartii</i>	3.0	tr.-13.1	Resistant varieties
1.0	Potato	Scab	<i>Actinomyces scabies</i>	1.8	1.5-2.6	Seed treatments
1.0	Tomato	Septoria blight	<i>Septoria lycopersici</i>	2.2	0.5-6.2	Copper sprays & dusts

\* General control measures such as clean seed, cultural practices, rotation, sanitation, etc.

† 3 years only.

‡ 5 years only.



factor in the simple estimate of disease index, but the extreme extent of 10 years fluctuations in per cent disease loss, may be seen in the column of ranges in Table II. Since the losses given were obtained originally from individual state losses weighted for production, diseases which may be severe and highly important in certain states or areas may not be considered important on a national basis. The conspicuous example here is black rot of grapes which causes a 4 per cent loss in the states east of the Rocky Mountains, but since the disease is insignificant in California which pro-

TABLE III  
ADDITIONAL DISEASES PROBABLY OUTSTANDING, BETTER CONTROL  
BY FUNGICIDES INDICATED

Host	Disease	Organism	Present major control measure
Citrus	Scab	<i>Elsinoe fawcettii</i>	Copper sprays
Citrus	Melanose and stem end rot	<i>Diaporthe citri</i>	Copper sprays
Sorghum	Covered kernel smut	<i>Sphacelotheca sorghi</i>	Copper, mercury, seed treatments, resistant varieties
Peanut	Leaf spot	<i>Cercospora personata</i>	Sulphur dust
Rice	Brown spot	<i>Helminthosporium oryzae</i>	Resistant varieties, seed treatments
Celery	Late blight	<i>Septoria apii</i>	Copper sprays or dusts
Cabbage	Black leg, black rot	<i>Phoma lingam</i> , <i>Bacterium campestris</i>	General, seed treatments
Cucurbits	Anthracnose	<i>Colletotrichum lagenarium</i>	Seed treatments, copper sprays
Cucurbits	Downy mildew	<i>Peronosplasmopara cubensis</i>	Copper sprays
Many crops	Root knot	<i>Heterodera marioni</i>	Resistant crops, soil disinfestation

duced 88 per cent of the grapes, as seen in Table I, the disease becomes of minor importance on a national scale. It should be pointed out again that these losses are based on the amount of disease present following the customary control measures. Thus certain well known diseases commonly emphasized will not be found in Table II; this is a tribute to our disease control program which has notably reduced their ravages. Perhaps the best example here is bunt of wheat which, because of organic mercury and copper carbonate seed treatments and resistant varieties, causes only an average of 1.3 per cent of loss. Late blight of potato would undoubtedly stand much nearer the top of Table II were it not for the relative effectiveness of Bordeaux mixture.

Important crops for which there are no definite Plant Disease Survey estimates are: tame hay, citrus, soybeans, sorghums, and peanuts, while other common crops omitted are: rice, flax, lettuce, onions, celery, the

crucifers, and cucurbits. A supplementary list of diseases on some of these crops which are considered probably outstanding and which are more or less controllable by fungicides is given in Table III in order of crop value. Primarily storage and transit diseases are not given in Tables II or III chiefly because of lack of data. It would be desirable to include important diseases of ornamentals and timber crops as well as major diseases on a world wide basis but at present estimates are not available.

#### USE OF FUNGICIDES

The estimated yearly consumption in the United States of the three most important groups of fungicides, namely copper, sulphur, and mercury, are given in Table IV, as derived from "Agricultural statistics, 1942" (13),

TABLE IV  
ESTIMATED YEARLY CONSUMPTION OF COPPER, SULPHUR, AND MERCURY FUNGICIDES IN UNITED STATES

Fungicide	Thousands of pounds
Copper sulphate as Cu	21,000
Copper carbonate, basic as Cu	1,500
Copper oxide as Cu	1,000
TOTAL COPPER	23,500
Sulphur dust	110,000
Wettable sulphur	5,000
Lime sulphur 11,000,000 gallons or as S	27,000
TOTAL SULPHUR	142,000
Mercuric chloride as Hg	59
Mercurous chloride as Hg	8
Organic mercury as Hg	85
TOTAL MERCURY	152

Roark (8), and other sources. The estimates cover the period 1941 to 1944. Specific information on the consumption of formaldehyde for plant diseases and on the newer organic fungicides was not available.

A detailed estimate of the specific uses for the copper, sulphur, and mercury fungicides has also been derived from data obtained from Roark (8) and through the courtesy of Dr. John W. Roberts and Dr. S. P. Doolittle of the Division of Fruit and Vegetable Crops and Diseases, U. S. Department of Agriculture. The data are presented in Table V. It is interesting to note here that the potato and apple crops account for more than half the consumption of copper, apple more than half of sulphur, and potato more than one-fourth of the mercury. In considering the total losses caused by insects, Hyslop (5) has also included the cost of control measures. Had this been done in the present case, and the cost of fungicides added, it will be seen that in particular late and early blights of potato,

apple scab, peach brown rot, and peanut leaf spot would be considered even more outstanding.

The final use or application of a fungicide is primarily a question of

TABLE V  
PRINCIPAL USES FOR COPPER, SULPHUR, AND MERCURY FUNGICIDES IN UNITED STATES  
DERIVED FROM ESTIMATES BY THE U. S. DEPT. OF AGRICULTURE\*

Crop	Diseases or use	% of total fungicide
<b>A. COPPER</b>		
Potato	Late and early blights (ESC)	43
Apple	Arsenical safener, blotch & bitter rot (S)	19
Peach	Brown rot (Cal.) and leaf curl	5
Grape	Black rot (EM)	4
Citrus	Scab and melanose (Fla.)	3
Tomato	Early and Septoria blights	3
Apricot	Blight and brown rot (P)	2
Celery	Leaf spots	1
Pear	Scab and leaf blight	1
Almond	Blight and leaf spot (Cal.)	1
Pecan	Scab and leaf disease (S)	1
Sugar beet	Leaf spot (M)	1
Home gardens & orchards	General disease control	1
Other crops	—	3
Vegetable & other seed	Seed treatment	7
—	Soil amendment	3
<b>B. SULPHUR</b>		
Apple	Scab (EC)	62
Peach	Brown rot and scab (ECS)	17
Peanut	Leaf spot (S)	7
Grape	Powdery mildew (Cal.)	3
Home gardens & orchards	General disease control	3
Pecan	Leaf curl; rust (Cal.)	2
Pear	Scab and leaf blight (ES)	1
Cherry	Leaf spot and brown rot (EC)	1
Shade trees & ornamentals	—	1
—	Soil fungicide amendment	3
<b>C. MERCURY</b>		
Cereal	Seed treatments	48
Potato	Seed treatments	26
Cotton	Seed treatments	18
Vegetables	Seed treatments	8

Specific regions in parentheses: E, East; C, Central; M, Midwest; S, South; P, Pacific.

\* Courtesy of Drs. John W. Roberts and S. P. Doolittle.

economics and regardless of the amount of disease and effectiveness of the treatment it will be used only "if it pays." In general, the greater the farm value per acre (see Table I) the more expensive the fungicide treatment may be. Because of high labor costs and more material, field sprays and dusts are more expensive than seed treatments and are thus limited to the

high value per acre crops. It is probable that no spray or dust would find a use on the low-acre value grain crops; on the other hand, seed treatments are being used profitably. Sprays and dusts, however, will ordinarily be used if necessary on the several hundred dollar per acre tree crops and on the more valuable vegetables. New fungicides which are no more efficient may replace older and more or less adequate fungicides because of such desirable factors as greater ease of handling and lessened toxicity hazard to humans.

### CONCLUSIONS

Thus from an examination of the outstanding plant diseases, the present control measures and their efficiency, together with farm values per acre and current consumption of fungicides, some appreciation may be gained of those diseases promising a favorable use for new or improved fungicides. Likewise, an opinion may be formed on those diseases for which it is most desirable to develop standard laboratory or greenhouse methods of testing. It is, of course, to be noted that virus and vascular wilt diseases do not yet, with our present knowledge, indicate a successful use for chemical control. It should be again emphasized that these points of view are on an overall or national basis, and that certain diseases of considerable importance in certain areas, which are either actual or potential consumers of large amounts of fungicide, may not have been included.

Foremost among the outstanding diseases are the complexes of root, stalk, and ear rots of corn, and seedling blight and boll rot of cotton (3), and apple scab. In the case of the corn disease complex, it is probable that only the seedling blight stage is amenable to control by seed treatment. However, the need is clearly shown for more efficient fungicides for seed treatment of corn and cotton, while the improvement and standardization of existing laboratory and greenhouse methods for the rapid evaluation of such chemicals is essential. Despite the many years of research and volume of fungicide used on apple scab control, it would appear that there is still need here for a more efficient fruit and foliage fungicide or combination of fungicides and insecticides. Although a greenhouse method for apple scab has been developed (4), further elaboration to handle large numbers of test samples would be desirable. A fungicide, or combination fungicide and insecticide that would control tip and hopper burn of potatoes and flea beetles, as well as late blight, is also needed. However, the efficiency of Bordeaux mixture in the control of late blight, despite its injuriousness, is sufficiently marked so that it will not be readily displaced. A satisfactory rapid greenhouse method of evaluating fungicides to control late blight has already been developed (7). The smuts of oats do not appear to be as well controlled as bunt of wheat and more efficient seed treatments or more general use of existing ones is indicated. A stand-

ard method for evaluating fungicides is available for the oat smuts (2). Although a large volume of sulphur is used against brown rot of peaches and cherries, the control could be improved by a better foliage fungicide. The causal organism, *Sclerotinia fructicola*, is the most common fungus used in the standard laboratory slide-germination method of testing fungicides (1). Fire blight of pears and apples is the most outstanding bacterial disease, but to date little progress has been made in controlling it with the existing sprays or dusts. Attempts to control early and Septoria blights of tomato with copper sprays and dusts have not been particularly successful. A greenhouse method of evaluating such fungicides is available (7); also, the fungus *Alternaria solani* is commonly used in the slide-germination method. Adequate estimates of the loss caused by peanut leaf spot are not available but it is evident that a large volume of sulphur is consumed for this purpose. More efficient fungicides could probably be used.

Most of the more or less standardized methods which are available for evaluating fungicides in the laboratory or greenhouse have been mentioned above. It is thus apparent that for most important diseases, specific methods have not been developed; however, this may not be necessary in all cases. A certain method may adequately represent a group of related diseases, for example the tomato late blight may well be indicative of most downy mildews, or bunt of wheat or the covered and loose oat smuts of all seedling infection smuts (3). Indeed, it has been hoped that the recently developed snapdragon rust method (6) will be representative of rusts in general. Usually, however, it is preferable that the most important disease of a group be developed, if practical, as the test method.

It is to be regretted that more detailed and accurate annual estimates are not available of the losses caused by different plant diseases in the different states, and for the consumption of the different fungicides by states and by diseases. If more specific information were available a detailed factual presentation of this subject and also one less biased by personal considerations, would be possible. Furthermore a fund of basic information would be at hand not only for the plant pathologists but also for the manufacturers of fungicides and others interested. A beginning could be made on the elucidation of the natural laws involved with benefit to all concerned.

#### SUMMARY

In order to facilitate research on new fungicides and specifically the selection of rapid methods for evaluating them in the laboratory and greenhouse, it is necessary to know the outstanding plant diseases. A tentative method is presented for determining the importance of the disease loss on an overall or national basis. An index is obtained from the product of the logarithm of the estimated annual per cent loss (from the Plant Disease

Reporter) and the logarithm of the farm value expressed in units of \$100,000.

A table is given of the 50 leading agricultural crops of the United States together with the farm value, acreage and farm value per acre, as well as the five leading states. The 36 outstanding diseases obtained by the above procedure are recorded, together with average annual loss, 10-year range in fluctuation and present major control measures. Tables are also presented showing the estimated annual consumption of fungicides by chemicals and by crops and diseases. From this the more outstanding potential uses may be seen for new or improved fungicides, and hence the diseases for which it is desirable to develop test methods. Among the outstanding diseases where need for better fungicides are indicated are: (a) seed treatments—corn and cotton seedling blights; and oat smuts; (b) sprays and dusts—apple scab; potato tip burn and late blight; peach and cherry brown rot; pear blight; peanut leaf spot; and tomato blights. More or less adequate laboratory or greenhouse methods for fungicide evaluation are available in certain of the cases cited while development and standardization is necessary for most other important diseases.

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## GERMINATION OF SEEDS OF PANICUM ANCEPS MICHX.

HELEN R. GARMAN AND LELA V. BARTON

### INTRODUCTION

*Panicum anceps* Michx. shows promise for use in soil conservation work in southern United States. Some difficulty, however, has been encountered in germinating the seeds. In the many published reports on special treatments for inducing germination in various grass seeds, daily alternation of temperature, low-temperature pretreatment, light, potassium nitrate, and concentrated sulphuric acid are described as effective methods. These results suggest two possible factors as causes of delayed germination—dormant embryos and impermeable seed coats.

Experience with seeds of the genus *Panicum* has indicated that one or both of these causes was involved. Akamine (1) treated seeds of *Panicum prolutum* with concentrated sulphuric acid for 6 to 12 minutes and obtained increased germination. He found that soaking in water was effective several months after harvest but that freshly-harvested seeds did not respond to the treatment. He also gave seeds one week moist pretreatment at 4° C. with favorable results. He believed that trapped carbon dioxide escaped from the seeds upon transfer from low to high temperature, thus enabling germination to take place. Edwards (4) found that rubbing the seeds of *Panicum colonitum* in a mortar with a pestle covered with sand-paper increased the germination from 0 to 40 per cent. He concluded that the lemma and palea were impermeable and therefore prevented germination. Piacco (5) removed coats of seeds of *Panicum* sp. and obtained 91 per cent germination. Soaking seeds for eight days in water also gave him good results. Toole (6) prechilled *Panicum obtusum* seeds for 14, 28, and 56 days at 3°, 5°, and 10° C. She then germinated the treated seeds at alternating temperatures of room temperature to 35° C. Her results indicated that prechilling the seeds at 3° C. for 28 days gave good germination. She also obtained satisfactory results by acid treating the seeds. Cullinan (3) used an alternating temperature of 15° C. (in light) to 35° C. (in dark) to germinate seeds of *Panicum maximum*. Burton (2) has pointed out that, in addition to delayed germination, the large percentage of empty florets and ergot are factors which hinder the establishment of southern grasses from seed. Although none of these reports were concerned with the species *anceps*, they have indicated the probable difficulties to be encountered. The present tests were undertaken to determine the conditions necessary for the germination of seeds of *Panicum anceps* and, if possible, to recommend a practical procedure for use on a large scale.

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## EXPERIMENTAL PROCEDURE AND RESULTS

The fruit of *Panicum anceps* is a caryopsis or grain. The seed, however, is so closely associated with the pericarp or ovary wall of the fruit, that, for all practical purposes, the caryopsis may be regarded as a seed.

Seeds were obtained from Mr. A. C. Mathews of the U. S. Dept. of Agriculture Soil Conservation Service, Chapel Hill, North Carolina. Two lots were used, Lot A harvested August 1943 and Lot B harvested in 1944. The grains were examined under a binocular microscope to see whether all of them contained well-developed seeds and embryos. This examination was necessary to determine what germination percentage could be expected from effective treatment methods. Many of the fruits were light-colored, i.e., cream-colored or green instead of the light brown color usually associated with mature seeds. This was especially true of the 1944 crop. Two samples of 100 grains each were taken without any selection and examined with the following results: Lot A, 15 and 16 and Lot B, 29 and 30 grains with undeveloped seeds. All of the well-developed seeds of both lots contained apparently sound embryos. Thus effective germination methods should have resulted in approximately 85 per cent germination for Lot A and 70 per cent for Lot B.

*Lot A. 1943 crop.* As soon as received, samples of this lot of seeds were placed on moist filter paper at constant temperatures of 15°, 20°, 25°, and 30° C., and daily alternations of 10° to 20° C., 10° to 30° C., 15° to 30° C., 20° to 30° C., and in light and dark at room temperature. A planting was made in soil in the greenhouse at the same time. The highest number of seedlings obtained was 9 per cent at 25° C. A few seeds also germinated in light at room temperature and in soil in the greenhouse as well as at daily alternations of 10° to 30° C. and 15° to 30° C. These results indicated a lack of a specific temperature requirement for germination.

Samples were then treated with concentrated sulphuric acid for periods of 1, 5, 10, and 30 minutes to determine whether impermeable coats were responsible for failure to germinate. The first three periods were without effect but a slight increase in germination (up to 15 per cent) at 25° C. followed sulphuric acid treatment for 30 minutes. The seeds were soaked in acid for the specified time after which they were plunged quickly into a large amount of water to prevent heating. This was followed by a thorough washing in running water to remove the acid.

Since neither germination temperature nor coat treatment alone brought about germination, other methods were tried. Seeds were treated with 71.0 per cent  $H_2SO_4$ , 0.2 per cent  $KNO_3$ , 35 per cent  $NaOH$ , 50 per cent  $HCL$ , and 0.025 per cent  $HgCl_2$ , several concentrations of dioxygen and ether, water at various temperatures, and dry heat, all without effect.

Then a series of treatments was begun, combining coat treatments with low temperature pretreatment. From some of the seeds all the enclosing

fruit structures were removed by rubbing lightly between two squares of fine sandpaper. Others were treated for 15, 30, or 45 minutes with concentrated sulphuric acid. Still others were placed in a glass bottle and shaken vigorously by an electric motor, a method that has proved efficacious for certain hard-coated seeds. Control lots were grains just as they came from the plants.

Samples of all of these were placed on moist filter paper for pretreatment at 5° C. or planted in soil in flats placed in a board-covered frame out-of-doors. One-half of the seeds planted in soil were sterilized with 0.025 per cent  $\text{HgCl}_2$  before sowing.

From Table I it will be seen that low temperature pretreatment for four to eight weeks resulted in increased germination upon transfer to a

TABLE I

EFFECT OF COAT AND LOW TEMPERATURE PRETREATMENT ON GERMINATION OF *PANICUM ANCEPS* (1943 CROP) AT A DAILY ALTERNATING TEMPERATURE OF 15° TO 30° C.  
100 SEEDS EACH

Seed treatment		Per cent germination after weeks at 5° C.			
		0	2	4	8
None		1	2	32	58
Soaked in conc. $\text{H}_2\text{SO}_4$	15 min.	30	35	69	58
	30 min.	38	25	28	19
	45 min.	1	3	2	0
Shaken	20 min.	1	2	73	87
	40 min.	1	5	88	93
Pericarp removed		71	35	37	31

daily alternating temperature of 15° to 30° C. Similar results were secured when the seeds were removed from 5° C. after eight weeks and planted in soil in the greenhouse. Also it will be noted that coat treatment, either by concentrated sulphuric acid for 15 minutes or by shaking, enhanced the germination values obtained after pretreatment for four weeks at 5° C. If the pericarps were removed 71 per cent germination followed at 15° to 30° C. without any further pretreatment. Such seeds planted in soil, however, failed to develop due probably to the action of decay organisms. Many of these seeds were injured slightly in removing the fruit coats. Sterilization with  $\text{HgCl}_2$  failed to remedy this. Similarly, pretreatment of these naked seeds at 5° C. for two, four, or eight weeks reduced their germination capacity at 15° to 30° C. This also was due to their susceptibility to mold.

Plantings were made in May, July, and September 1944 and in January 1945 in flats placed in a board-covered frame. Neither sulphuric acid nor mercuric chloride treatments were effective. The best seedling production

was obtained from untreated entire grains. However, the percentages were not high. Twenty-four, 2, 33, and 16 per cent of the seeds germinated by the middle of June 1945 from sowings made the preceding May, July, September, and January respectively. The low figure from the July planting was most likely due to the heat immediately following planting.

*Lot B. 1944 crop.* Since shaking seeds of the 1943 crop appeared to increase their germinative power, this process was repeated with seeds of the 1944 crop. However, no advantage was gained in the latter case.

Again low temperature pretreatment on moist filter paper at 5° C. for eight weeks brought about good seedling production upon transfer to soil in the greenhouse. Periods of two and four weeks were much less favorable. Seedling production in soil was 6, 7, and 66 per cent following pretreatment for two, four, and eight weeks respectively at 5° C. It will be recalled that approximately 70 per cent of the seeds of this crop possessed good embryos. Thus eight weeks at 5° C. prepared about 100 per cent of the good seeds for germination in soil. These seedling production tests were made with duplicates of 200 seeds for each test. Control lots planted in soil in the greenhouse without pretreatment yielded only 1 per cent germination.

In the instance reported above, the moist seeds were transferred directly from 5° C. to moist soil. It was of interest to determine whether seeds could be pretreated on a moist medium and then dried before sowing and still retain their ability to produce seedlings. This would have an important bearing on the practical application of the germination method. Consequently other lots of seeds were removed from moist filter paper after eight weeks at 5° C. Controls were planted in soil immediately. Other lots were placed on dry filter paper at room temperature where they were allowed to remain for periods of one day, one week, and two weeks before planting. None of the three drying periods affected the subsequent seedling production in soil, where 54, 61, 58, and 58 per cent were obtained after no drying and drying for one day, one week, and two weeks respectively. The appearance of three of the flats of seedlings is shown in Figure 1. Again duplicates of 200 seeds were planted in each flat. All of the seeds were pretreated on moist filter paper at 5° C. for eight weeks prior to planting in soil and removed from 5° C. at the same time. The seeds in flat A were planted on August 30, 1945 immediately upon removal from 5° C. Seeds in flats B and C were planted on September 6, and September 13, 1945 after having dried in the laboratory for one to two weeks. In each case, the photograph was taken 25 days after the seeds were planted in soil. This means that all of the seeds had the same length of time to germinate and the seedlings to develop before being photographed. There seems to be an indication of less vigor of growth with increased period of drying pretreated seeds before planting even though the germination percentage

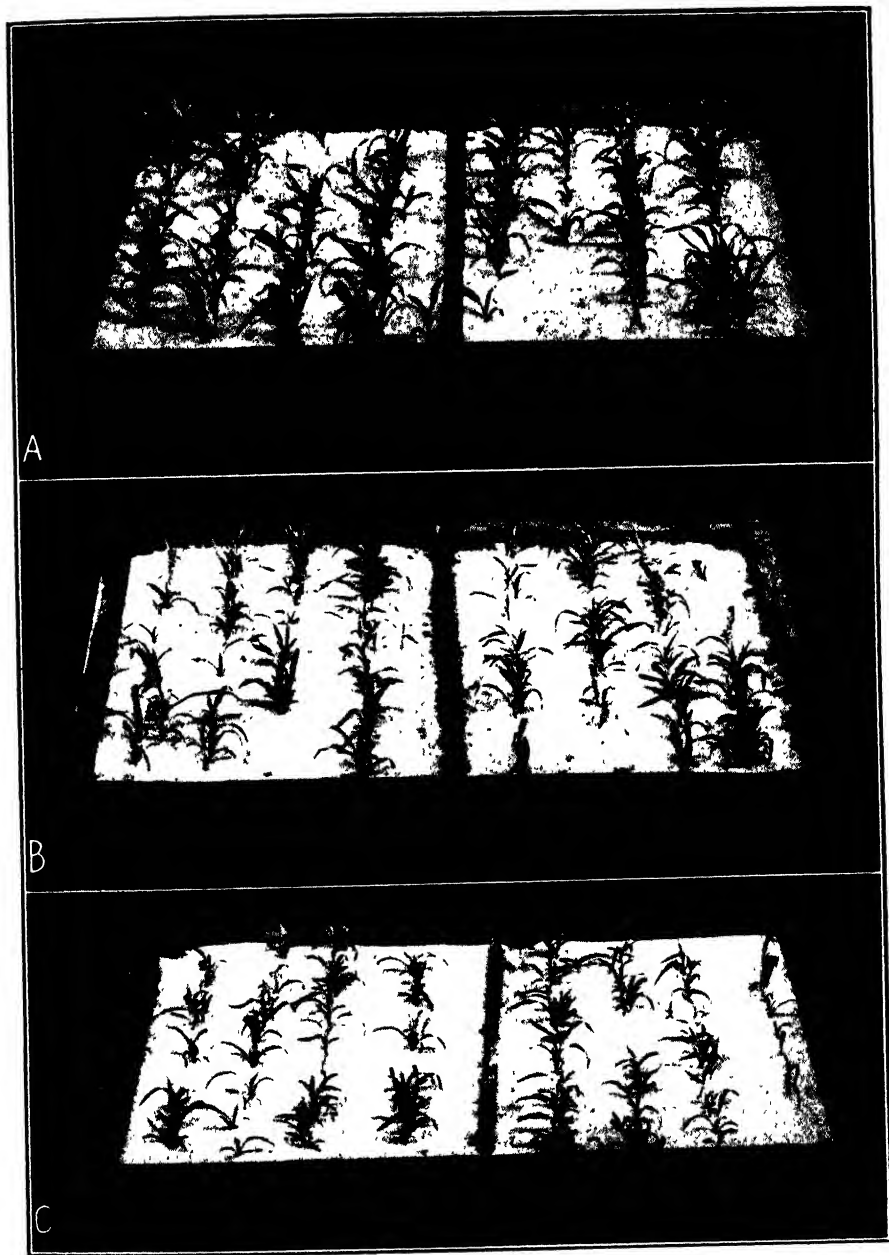


FIGURE 1. Seedlings from plantings of *Panicum anceps*, 1944 crop, in the greenhouse after eight weeks' pretreatment on moist filter paper at 5° C. A. Seeds planted immediately upon removal from 5° C. B and C. Seeds dried for one and two weeks respectively after pretreatment at 5° C. before planting in soil.

was unaffected. This is shown by the decreased size of the plants in flats B and C as compared with A and of those in flat C as compared with B. Emergence of seedlings from the soil took place in seven days in flat A, in six days in flat B, and in 11 days in flat C.

Eight additional lots of 200 seeds each were removed from moist filter paper after 13 weeks at 5° C. Again two lots were planted in the greenhouse immediately where 58 per cent of them produced seedlings. The other six lots were dried at room temperature as before but the drying time was extended to two months. After one month of drying, the low temperature pretreatment effect as measured by the per cent seedling production in the greenhouse had not diminished. However, pretreated seeds dried two months before planting were reduced in germination to 22 per cent.

#### SUMMARY

The most effective pretreatment for seeds of *Panicum anceps* was eight weeks' moist storage at 5° C. Well-developed seeds thus treated gave approximately 100 per cent germination upon transfer to soil in the greenhouse. The germination capacity of pretreated seeds was not affected by drying in the laboratory for periods up to one month but was reduced more than one-half by drying as long as two months before planting.

Removal of the fruit coats, treatment with concentrated sulphuric acid, or vigorous shaking seemed to increase the germination somewhat for one crop of seeds but showed no advantage for a second crop.

The occurrence of empty grains or grains with undeveloped seeds in some lots is a factor in poor germination.

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## HISTOLOGICAL EFFECTS OF SESAMIN ON THE BRAIN AND MUSCLES OF THE HOUSEFLY

ALBERT HARTZELL AND ELEANOR WEXLER

The histological effects of pyrethrum and sesame oil, an activator of pyrethrum, on the central nervous system and muscles of the housefly (*Musca domestica* L.) have been described by Hartzell (2). The reader is referred to the previously named publication for a review of the literature on activation as related to housefly sprays and a discussion of the effects on the nerves and muscles of the housefly of some of the better known activators and poisons used in sprays, such as sesame oil, piperine, Lethane 384, Thanite, D. H. S. Activator, pyrethrum, rotenone, and DDT or 2,2 bis-(*p*-chlorophenyl)-1,1,1-trichlorethane. Flies rendered moribund by pyrethrum (0.1 per cent pyrethrins) showed clear spaces in the brain tissue and dissolution of the fiber tracts, while with sesame oil (50 per cent) there was vacuolation around the large nerve cells. When sesame oil (10 per cent) and pyrethrum (0.05 per cent) were combined, the nerve fibers were not only destroyed, but the larger nerve cells were highly vacuolated resulting in almost complete lysis of the tissue. The stains used in the aforementioned study were hematoxylin and eosin Y for all spray treatments. In addition, Bodian's method was used on flies sprayed with pyrethrum.

The histological effects of pyrethrum on muscle tissue in moribund flies which had been killed and fixed in formalin and stained with Delafield's hematoxylin and eosin Y showed the chromatin of the nuclei clumped into dense rod-like masses, and occasional fenestration of the tissue, while with sesame oil the muscle nodes and Krause's membrane were greatly accentuated. When sesame oil and pyrethrum were combined, the muscle bands stood out very prominently.

Pure sesamin was not available at the time the previous study (2) was made, so that it was not possible to determine whether the effects produced by sesame oil on nerve and muscle tissues were due to its sesamin content or to other constituents. The writers are indebted to Dr. H. L. Haller of the Bureau of Entomology and Plant Quarantine of the United States Department of Agriculture for a 2-g. sample of pure sesamin, which was used in this study.

The present investigation was undertaken to determine whether pure sesamin is a neuro-muscular poison for houseflies which may be detected by histological methods. Comparisons have been made with other neuro-muscular poisons alone or in combination, namely pyrethrum, sesame oil, sesame oil and pyrethrum, and a proprietary product designated as Improved Pyrin 20, known to contain sesame oil extractive and pyrethrum.

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## MATERIALS AND METHODS

Adult houseflies, five days old, of both sexes were sprayed under regular Peet-Grady test (3) conditions with various concentrations of the poisons and activators in a highly refined kerosene ("Deo-base"). Moribund individuals which were capable of slight movements on probing, but unable to fly, were collected after 10 minutes. Immediately upon collecting, the abdomens were punctured by a needle to allow complete permeation of the tissues and the flies were placed whole in a 10 per cent solution of formalin for at least 24 hours for killing and fixing. They were dehydrated whole in a series of alcohols according to the following schedule: 80 per cent alcohol, one and one-half hours, 95 per cent alcohol, two changes one hour each; absolute alcohol, two changes one hour and one-half hour each. Following this, they were transferred to a mixture of equal parts of xylol and absolute alcohol one-half hour, and finally cleared in xylol for one hour. Infiltration was accomplished by first allowing the tissue to stand overnight in a mixture of xylol and paraffin (m.p. 50° to 52° C.) in a constant-temperature oven kept at 54° C. It was then placed in two baths of harder paraffin (60° to 62° C.) and imbedded in a fresh quantity of the same.

Sections were cut at 5 $\mu$  and stained in Delafield's hematoxylin for 25 seconds and in eosin Y for one minute. Controls were subjected to the same treatment for comparison (1).

Two per cent sesamin was dissolved in 40 per cent acetone and 60 per cent "Deo-base." At this concentration a knockdown of 74 per cent in 10 minutes and a kill of 27 per cent in 24 hours under Peet-Grady test conditions were obtained.

Pyrethrum was dissolved in a highly refined kerosene to give a concentration of total pyrethrins of 1 mg. per cc., or approximately 0.1 per cent.

Improved Pyrin 20, obtained from John Powell & Co., Inc., New York, New York, was used at a concentration of 5 per cent. According to the manufacturer this product contains 800 mg. of pyrethrins per 100 cc., 3 g. sesame oil extractive with sesamin content of 15 to 25 per cent and a co-solvent of a high molecular weight hydrocarbon.

## RESULTS

## EFFECTS ON BRAIN TISSUE

The principal effect of sesamin on the brain of the housefly in moribund individuals was the vacuolation of the tissue around the larger nerve cells and the destruction of the fiber tracts, especially in the region of the corpus centrale (Fig. 1, compare A and B). The effect of pure sesamin is similar to that of sesame oil (50 per cent) as previously reported by Hartzell (2, p. 447, Fig. 1 E).

When sesamin (0.25 per cent) and pyrethrum (0.025 per cent) were combined the tissue showed vacuolation and almost complete lysis (Fig. 1 C).

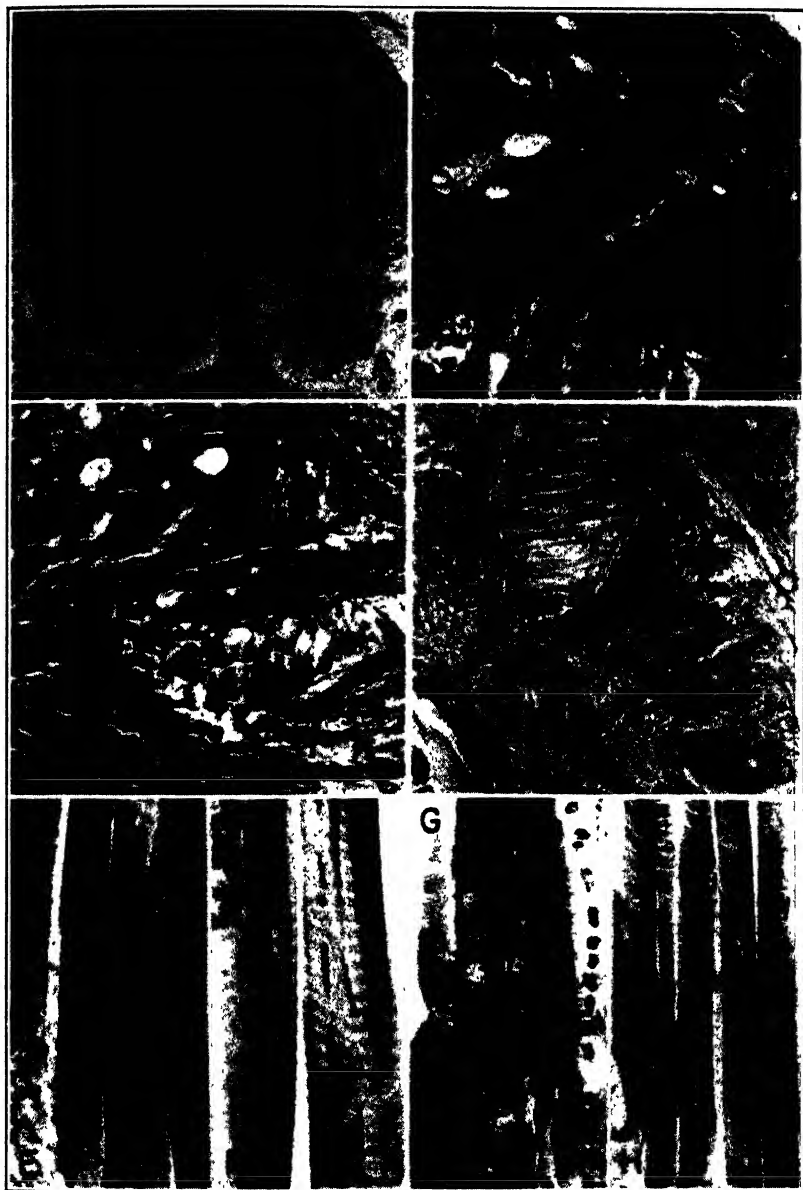


FIGURE 1. Sections of brain and muscle tissues of the housefly, fixed in formalin and stained with hematoxylin and eosin Y ( $\times 333$ ); 10 min. after spraying, except checks. Brain (fiber tracts and corpus centrale region). A. Check. B. Effect of sesamin, vacuolation of large nerve cells. C. Sesamin and pyrethrum, vacuolation and lysis of tissue. D. Improved Pyrin 20, prominence of nerve fibers, less dissolution, otherwise similar to C. Muscles. E. Check. F. Effect of sesamin, nodes and Krause's membrane accentuated. G. Sesamin and pyrethrum, clumped nuclear chromatin. H. Improved Pyrin 20, similar to G.



The effects of Improved Pyrin 20 (5 per cent) were similar to the combined effect of sesamin and pyrethrum except that the nerve fibers stood out more prominently due to separation (Fig. 1 D) and showed less dissolution than with sesamin and pyrethrum.

#### EFFECTS ON MUSCLE TISSUE

The foremost effect of sesamin in the striated muscles of the housefly was the accentuation of the muscle nodes and Krause's membrane (Fig. 1, compare E and F). When sesamin and pyrethrum were combined (Fig. 1 G) the typical effect was a clumping of the chromatin of the nuclei into rod-like masses, similar to that caused by pyrethrum alone.

The effect of 5 per cent Improved Pyrin 20 on striated muscle (Fig. 1 H) was similar to that of sesame oil and pyrethrum as reported by Hartzell (2, p. 451, Fig. 4 F).

#### DISCUSSION

The effects of pure sesamin on the brain and striated muscles of the housefly are similar to that of sesame oil at a high concentration. Combinations of sesamin and pyrethrum showed similar effects on the brain and muscle tissues to combinations of sesame oil and pyrethrum. There is thus histological evidence that the active principle of sesame oil is sesamin.

Activation appears to be due to the destruction of two tissue components, namely the fibers and nerve cells. Pyrethrum destroys the fiber tracts while sesamin causes vacuolation of the larger nerve cells.

The effect of Improved Pyrin 20 is similar to that of the combined effect of sesamin and pyrethrum for both muscle and brain tissues, except that in the last named tissue the fibers were more prominent and showed less dissolution than in flies sprayed with Improved Pyrin 20.

#### SUMMARY

Sesamin was found to produce characteristic effects on the brain and striated muscles in moribund houseflies in a preliminary histological study in tissues stained with Delafield's hematoxylin and eosin Y.

The principal effect was the vacuolation of the larger nerve cells of the brain, and the accentuation of the nodes and Krause's membrane in the striated muscles.

The effects of pure sesamin on brain and muscle tissues of the housefly were in general similar to those obtained with sesame oil.

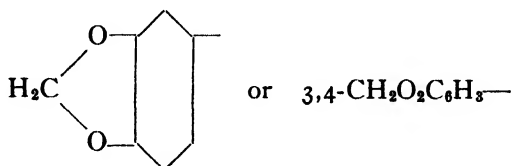
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# INSECTICIDAL THIO ETHERS DERIVED FROM SAFROLE, ISOSAFROLE, AND OTHER ARYL OLEFINS

EDWARD A. PRILL, ALBERT HARTZELL, AND JOHN M. ARTHUR

A number of insecticidal compounds discovered in the past few years contain the 3,4-methylenedioxyphenyl residue in their structures. This residue may be represented by the structural formula or by the condensed formula shown below.



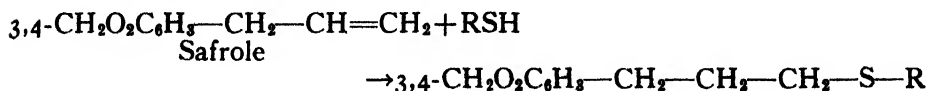
Synerholm and others (17, 18) have recently reported on the preparations and tests of a number of such compounds and have reviewed (17) the literature. Very recently another insecticidal compound which contains this residue in its structure and which is said to be piperonylcyclohexenone,<sup>1</sup> has attained commercial importance.

Safrole, which occurs as a major component in certain essential oils, particularly those from certain of the Lauraceae, is probably the most important primary natural raw material for preparing intermediates for the syntheses of more complex compounds containing the methylenedioxyphenyl residue in their structures. Thus safrole may be easily isomerized into isosafrole, and this in turn oxidized to piperonal or to piperonylic acid. It is a distinct advantage to be able to prepare insecticidal compounds directly from safrole or isosafrole.

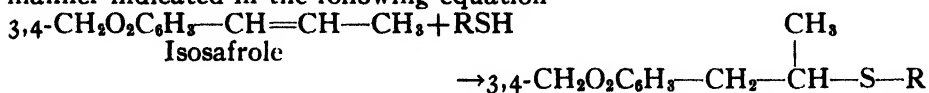
Both safrole and isosafrole were found to react very readily with any of a number of sulphydryl compounds and the products produced were found in most cases to be especially active insecticides toward houseflies and also toward certain agricultural insects. Products made with certain other aryl olefinic compounds in place of safrole or isosafrole were in most cases relatively inactive toward houseflies but were active toward certain agricultural insects. The reaction whereby olefinic compounds are chemically combined with sulphydryl compounds or mercaptans may be simply effected by having a trace of some organic peroxide present in the reaction mixture; and the reaction is known as the peroxide catalyzed addition reaction, or sometimes as the "abnormal addition" reaction of mercaptans and olefinic compounds (9, 10, 12). When safrole is used in the reaction

<sup>1</sup> An active ingredient of "Pyrenone No. 20" sold by Dodge and Olcott, Inc.  
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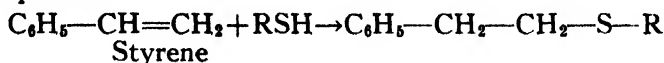
it is probable that the sulphydryl compound, RSH, is added across the olefinic double bond in the manner indicated in the following equation



This represents the formation of the expected "abnormal addition" reaction product. When isosafrole is used in the reaction it is probable that the sulphydryl compound is added across the olefinic double bond in the manner indicated in the following equation



The correctness of these equations has not been rigorously established and the possibility of any alternate manners of the additions of the —SR and the —H to the olefinic double bonds is not entirely excluded. Styrene, which is somewhat analogous to isosafrole in that it also contains an olefinic double bond in the same relative position to the benzene ring, has been definitely proven in two cases (10, 12) to react in the manner shown in the following equation



The other aryl olefins used, which comprised anethole, eugenol, and 2-vinylpyridine, presumably, react analogously to the above. A few related compounds made by another method will be discussed later in this paper.

Although many compounds containing a 3,4-methylenedioxyphenyl residue in their structures have been found active as insecticides or as synergists with pyrethrum, it would be unwarranted to assume that the mere presence of this residue in any organic molecule would impart insecticidal activity to it. Haller and others (5) cited several relatively inactive compounds containing this residue, such as safrole, piperonal, and ethyl piperonylate. In this laboratory a great number of types of 3,4-methylenedioxyphenyl compounds have been prepared and tested and while some of these were active, many others (unpublished results) were relatively inactive against houseflies. A few relatively inactive compounds containing both the methylenedioxyphenyl residue and sulphur are included in this paper for comparison with the active compounds.

It would appear that a methylenedioxy substituent on a benzene ring may contribute activity to a compound only if the compound also contains one or more other structural components of suitable types. Moreover, the introduction of still another structural component into an active compound of this type may sometimes increase the activity or it may tend to reduce

or nullify the activity. The proper solubility in lipoids also probably is important. This paper presents additional evidence for these ideas.

Several of the new compounds presented herein compare favorably with pyrethrins in toxicity. The compounds are divided into four classes and are presented in following sections of this paper.

#### PROCEDURE FOR TESTING ON HOUSEFLIES

*Peet-Grady method.* All of the compounds were tested on houseflies (*Musca domestica* L.) by the large group Peet-Grady method as recommended by the National Association of Insecticide and Disinfectant Manufacturers (14). Control tests with the regular Official Test Insecticide (OTI), supplied by the above Association, were performed on each batch of flies used. The regular OTI contains approximately 0.1 g. pyrethrins per 100 ml. (13). When possible, the test compounds were dissolved in "Deo-base," a purified petroleum distillate, without any admixed co-solvent. When the test compounds were not sufficiently soluble in "Deo-base" alone, they were dissolved in mixtures consisting of a small amount of acetone and "Deo-base." The specific cases where this was necessary will be mentioned later. Solutions of some of the compounds without admixed pyrethrins were tested. When so tested the knockdown was often very incomplete and since only the flies knocked down are collected, it is obvious that the kill can not be greater than the 10-minute (or in some cases the 15-minute) knockdown. One or more concentrations of each compound in admixture with 0.05, 0.025, or 0.0125 g. of pyrethrins per 100 ml. were tested.

*Pyrethrins.* The pyrethrins used for admixing with the test compounds were from a purified pyrethrum extract supplied by S. B. Penick and Company in the form of a concentrate containing 20 per cent pyrethrins. Analysis by the Seil method (16) showed that 55.5 per cent of the total pyrethrins was Pyrethrin I and 44.5 per cent was Pyrethrin II. Solutions containing 0.025 g. pyrethrins only per 100 ml. gave an average 10-minute knockdown of 84 per cent, and after adjusting the kills by means of probits (1) to an OTI kill of 50 per cent, an average adjusted 24-hour kill of 21 per cent. Solutions containing 0.05 g. pyrethrins only per 100 ml. gave an average 10-minute knockdown of 93 per cent and after adjusting the kills to an OTI kill of 50 per cent, an average adjusted 24-hour kill of 32 per cent. These kills are only slightly higher than those shown in the dosage-mortality curve given by Kilgore and others (11).

#### CLASS I PRODUCTS—PREPARATION AND TOXICITY TO HOUSEFLIES OF SAFROLE-MERCAPTAN AND ISOSAFROLE-MERCAPTAN PRODUCTS

*Safrole and isosafrole.* The safrole was of a pure grade and the isosafrole was of the practical grade from Eastman Kodak Company.

**Mercaptans.** Those mercaptans that were not readily available from commercial sources were prepared from the corresponding chloro or bromo compound and thiourea by adaptations of a standard laboratory method (19). After hydrolysis of the intermediary isothiuronium halide to the mercaptan, the cooled reaction mixture was made strongly alkaline with sodium hydroxide and any organic halide or disulphide was removed from the sodium mercaptide solution by extraction with ethyl ether. The solution was kept cold and strongly acidified with hydrochloric acid, and the mercaptan then extracted with ethyl ether. A dimercapto compound,  $\beta,\beta'$ -dimercaptodiethyl ether, was similarly prepared from  $\beta,\beta'$ -dichlorodiethyl ether and a liberal excess of thiourea. Some of the halides needed for making mercaptans were also prepared. The following outline gives the sources of the mercaptans. Reference to Table I will reveal the radical of the mercaptan involved.

- A. From commercial sources.
- B. From the corresponding bromo compound and thiourea.
- C. From nuclear substituted benzyl chlorides and thiourea.
- D. Various ether alcohols of the "Cellosolve" and "Carbitol" types were converted into the chlorides by means of thionyl chloride and pyridine. The chlorides were purified by distillation and were then reacted with thiourea.
- E. *n*-Hexyl alcohol, butyl "Cellosolve" and tetrahydrofurfuryl alcohol were each converted to its sodium derivative and this reacted with an excess of  $\beta,\beta'$ -dichlorodiethyl ether. The sodium derivative of tetrahydrofurfuryl alcohol was similarly reacted with an excess of trimethylene dibromide. In each case the monohalide was isolated by fractional distillation under reduced pressure. The yields in these cases were rather low. Each monohalide was then reacted with thiourea.
- F. The sodium derivative of the appropriate phenolic compound in absolute ethanol solution was reacted with an excess of ethylene dibromide, trimethylene dibromide, or  $\beta,\beta'$ -dichlorodiethyl ether. In each case the monohalide was isolated in good yield by fractional distillation under reduced pressure and was then reacted with thiourea.
- G. Furfuryl mercaptan was made from furfural by the method of Gilman and Hewlett (4).

**Preparation of safrole-mercaptan and isosafrole-mercaptan products.** Mixtures containing 0.1 mole of a monomercapto compound and 0.1 mole of safrole, mixtures containing 0.1 mole of a monomercapto compound and 0.1 mole of isosafrole, a mixture containing 0.1 mole of  $\beta,\beta'$ -dimercaptodiethyl ether and 0.3 mole of safrole, and a mixture containing 0.1 mole of this dimercapto compound and 0.3 mole of isosafrole were prepared and to

each mixture were added a few drops of ascaridole, which is an organic peroxide. The reaction mixtures containing the very volatile methyl and ethyl mercaptans were held in pressure bottles for about two weeks at room temperature for the reaction to take place. The addition reactions of *n*-butyl, *p*-tolyl and benzyl mercaptans with isosafrole were also thus effected at room temperature, and they could also be more rapidly effected at 100° C. For each of the other reactions, the reaction mixture in a flask fitted with a reflux condenser was held at about 100° C. for 48 hours, to bring about the reaction.

By the use of somewhat aged and presumably slightly autoxidized samples of safrole and isosafrole, it was also possible to effect the addition reactions with *n*-butyl mercaptan at 100° C. without any special addition of a peroxide.

At the termination of the reaction period, the reaction mixture was in each case mixed with low boiling petroleum ether, or ethyl ether when the compound was not soluble in the former solvent, and any remaining mercaptan washed out with aqueous potassium hydroxide. The organic solvent was then evaporated. When possible the product was fractionally distilled under reduced pressure. The boiling points of these compounds are given in Table I. Due to accidental loss of the very volatile methyl mercaptan the products made from it were not sufficient in amount to get a satisfactory boiling point. The compounds which were not sufficiently volatile to be distilled were purified by removal of any steam distillable impurity and any excess safrole or isosafrole by steam distillation. A possible impurity remaining in these might be a small amount of a disulphide resulting from oxidation of a high molecular weight mercaptan by air. The products so purified by steam distillation were free of any unpleasant odor. The yields of the various compounds were frequently in the neighborhood of 70 per cent of the theoretical.

In the processing of these products, steam distillation also may be employed for the compounds which were distilled under reduced pressure, either in lieu of the distillation under reduced pressure or subsequent thereto, in order to remove any malodorous impurities. Also, if desired, any unreacted safrole or isosafrole may be allowed to remain in the product since this ordinarily would not be objectionable.

It is probable that other catalytic agents, which also function similarly to organic peroxides by providing free radicals for initiating chain reactions between the reactants (7), may be used for the reactions. Such catalytic agents as lead tetraethyl in the presence of ultra-violet light (3) or in the presence of heat would be expected to be effective, but the removal of undesirable organometallic compounds from the product would require additional processing.

The boiling point of a distilled compound made from isosafrole and a

straight chain mercaptan was regularly lower than that of a compound made from safrole and the same mercaptan. It is a fairly general rule that the isomer with the more branched structure has the lower boiling point. If the formula of the product from safrole indicated in the previously given equation is correct, then the isomeric product from isosafrole must necessarily have a more branched structure than that from safrole.

The products were not analyzed. The manner of preparation really defines the products. The distilled products boil at temperatures consistent with the molecular weight of the expected compound. As previously mentioned, the products not distilled may possibly contain a small amount of relatively non-volatile impurity.

With one exception, all the compounds in this class were viscous oily liquids at room temperature. All were very soluble in "Deo-base" except those made from mercaptans containing a benzene nucleus, a tetrahydrofuran residue, a hydroxyl group or two sulphhydryl groups. In preparing fly spray solutions it was necessary in the latter cases to include a co-solvent with the "Deo-base." Five per cent of acetone, and often less, was sufficient.

*Toxicity to houseflies of safrole-mercaptan (Type I) and isosafrole-mercaptan (Type II) products.* The data are shown in Table I. In general, the compounds when in admixture with pyrethrins appear to exhibit synergistic action. The compounds tested as higher concentrations without admixed pyrethrins showed considerable paralyzing and killing ability. Types I and II are more or less parallel in activity throughout; Type II being the more active except in a few pairs of compounds. Of the compounds containing simple alkyl substituents, those containing a *n*-amyl or a *n*-hexyl substituent are the most active. Compounds containing simple aryl or simple aralkyl substituents are active but not outstanding.

When the substituent radical contains one or more ethereal oxygen atoms, that is, the compound contains O-ether as well as S-ether linkages, the compounds are more active. In these cases the substituent apparently may be fairly large without causing a marked decrease in activity of the compound, whereas, in the case of compounds containing simple alkyl substituents, the activity starts to decrease when the substituent is *n*-heptyl.

One of the most active compounds, which is especially practical for fly sprays because of its great solubility in "Deo-base," is the product of isosafrole and 2-(2-*n*-butoxyethoxy)ethyl mercaptan (compound 39). The product of safrole and the same mercaptan (compound 38) appears to be only slightly less active. Incidentally, the substituent radical in these is the same as the substituent radical of the thiocyanate in the insecticide, "Lethane 384." The data for compounds 38 and 39 also illustrate an advantageous use of these compounds in admixture with DDT [2,2-bis(*p*-

chlorophenyl)-1,1,1-trichloroethane] and a minimum amount of pyrethrins. DDT is an excellent insect killer but it gives a very slow knockdown. Spray solutions containing 0.025 g. pyrethrins and 0.1 g. DDT per 100 ml. gave inadequate 10-minute knockdowns. By halving the amount of pyrethrins and adding a small amount of compound 38 or 39, a more satisfactory knockdown and a higher kill were obtained. The chief practical advantage of the use of these compounds with DDT-pyrethrum mixtures seems to be due to synergistic action in respect to knockdown between the thio ether and pyrethrum.

Compounds 54 and 56 made with isosafrole are almost equally as effective as the above and the related compounds 53 and 55 made with safrole are somewhat less active. A number of other compounds containing etheral oxygen in the substituent radical are also quite outstanding.

In compounds 66 and 67 the 3,4-methylenedioxyphenyl residue and the thio ether linkage occurred twice. These compounds also are quite active.

It is especially apparent in the case of the more active compounds that there is synergistic action with pyrethrins in respect to both knockdown and kill. It would require about 0.16 g. of pyrethrins per 100 ml. when used alone to produce a AA grade fly spray (11, 13), that is, one that will give at least a 16 per cent higher 24-hour kill than given by the OTI. It is possible to prepare AA grade fly sprays containing 0.2 g. or less of one of the more active compounds and only 0.025 g. pyrethrins per 100 ml. The saving in the expensive pyrethrins is obvious.

Compounds 68, 69, and 70 are relatively inactive. Comparison of these with compounds 3 and 4 shows that the substitution of a hydrogen of the ethyl radical by a hydroxyl or butyryloxy group nullifies the activity of the compound in these cases.

## CLASS II PRODUCTS—PREPARATION AND TOXICITY TO HOUSEFLIES OF N-SUBSTITUTED AMIDES AND ESTERS OF THIO ETHER ACIDS

*Acid I.* A mixture of 81 g. of safrole, 46 g. of mercaptoacetic acid, and a few drops of ascaridole was held at about 100° C. for 48 hours. The mixture was then neutralized with aqueous sodium hydroxide and any water-insoluble oil (unreacted safrole) removed. On acidifying with hydrochloric acid the product precipitated as an oil which soon solidified. The yield was 110 g. The product, Acid I, has the probable formula:



After recrystallization from a mixture of ethyl ether and low boiling petroleum ether its melting point was 71° C.

*Neutral equivalent.* Calculated for  $\text{C}_{12}\text{H}_{14}\text{O}_4\text{S}$ : 254.3. Found: 252.5.



TABLE I  
TOXICITY TO HOUSEFLIES OF THIO ETHERS  
TYPE I—PRODUCT OF SAFFROLE AND RSH; PROBABLE FORMULA:  $\text{CH}_3\text{O}_2\text{C}_6\text{H}_4\text{—CH}_2\text{CH}_2\text{CH}_2\text{—S—R}$   
 $\text{CH}_3$   
 TYPE II—PRODUCT OF ISOSAFFROLE AND RSH; PROBABLE FORMULA:  $\text{CH}_3\text{O}_2\text{C}_6\text{H}_4\text{—CH}_2\text{CH—S—R}$

Compound No.	Compound		B. P. °C./mm.	RSH from*	Concn. g. per 100 ml.	Pyrethrins g. per 100 ml.	10-Min. knock-down %	24-Hr. kill %	OTI kill %**
	Type	Wherein R is:							
1	I	$\text{—CH}_3$ Methyl		A	2.0	.025	95	85	45
2	II				1.0	.05	95	67	37
3	I	$\text{—CH}_2\text{CH}_3$ Ethyl	146-148/2	A	1.0	.025	95	85	57
4	II		133-135/3		1.0	.025	95	86	57
5	I	$\text{—(CH}_2)_3\text{CH}_3$ <i>n</i> -Butyl	168-171/3	A	.4	.05	95	68	47
6	II		135-137/1		.4	.025	95	68	63
					1.0	.025	99	86	54
					.5	.025	99	58	46
7	I	$\text{—(CH}_2)_4\text{CH}_3$ <i>n</i> -Amyl	178-182/3	A	1.0	0	(66)†	49	45
					.4	.05	98	88	53
8	II		160-165/3		.4	.025	91	59	49
					1.0	0	(54)	34	45
					.4	.05	99	94	60
					.2	.05	98	82	60
9	I	$\text{—(CH}_2)_5\text{CH}_3$ <i>n</i> -Hexyl	184-188/2	A	1.0	0	78	65	45
					.4	0	71	58	63
					.4	.05	99	85	53
					.2	.05	95	80	53
					.4	.025	94	68	57
10	II		170-175/2		1.0	0	(59)	46	41
					.4	.05	98	97	60
					.2	.05	98	93	60
					.4	.025	95	82	50

TABLE I—(Continued)

Com- pound No.	Compound		B. P. °C./mm.	RSH from*	Concn. g. per 100 ml.	Pyrethrins g. per 100 ml.	10-Min. knock- down %	24-Hr. kill %	OTI kill %**
	Type	Wherein R is:							
11	I	—(CH <sub>2</sub> ) <sub>6</sub> CH <sub>3</sub> <i>n</i> -Heptyl	202–204/4		.4	.05	96	62	53
12	II		175–180/2	A	.4	.025	93	60	51
13	I	—(CH <sub>2</sub> ) <sub>7</sub> CH <sub>3</sub> <i>n</i> -Octyl	195–198/1	A	.8	.025	94	62	55
14	II		190–195/2		.8	.025	96	61	55
15	II	—CH(CH <sub>3</sub> )(CH <sub>2</sub> ) <sub>5</sub> CH <sub>3</sub> 1-Methylheptyl	178–182/1	B	.4	.025	96	73	53
16	I		160–190/3		2.0 1.0	.05 .05	99 98	96 89	55 63
17	II	<i>tert</i> -C <sub>8</sub> H <sub>17</sub> <i>tert</i> -Octyl isomers	155–200/2 (mostly 160–170/2)	A	2.0 1.0	.05 .05	98 97	87 58	55 63
18	I	—(CH <sub>2</sub> ) <sub>8</sub> CH <sub>3</sub> <i>n</i> -Decyl	204–208/1		.8	.025	97	65	61
19	II		197–203/1	A	.8	.025	98	80	61
20	I		(M.P. about 26°)	A	2.0	.05	96	69	54
21	II	—(CH <sub>2</sub> ) <sub>11</sub> CH <sub>3</sub> <i>n</i> -Dodecyl			2.0	.05	96	79	59
22	I	—CH—(CH <sub>2</sub> ) <sub>5</sub>   Cyclohexyl	158–165/2		1.0 1.0 .4	0 .025 .05	68 97 98	47 88 79	43 55 59
23	II		156–164/2		1.0	.025	92	63	55

TABLE I—(Continued)

Com- pound No.	Compound		B. P. °C./mm.	RSH from*	Concn. g. per 100 ml.	Pyrethrins g. per 100 ml.	10-Min. knock- down %	24-Hr. kill %	OTI kill %**
	Type	Wherein R is:							
24	I	—C <sub>6</sub> H <sub>5</sub> Phenyl		A	1.0 .5	.025 .025	95 93	86 60	55 57
25	II	—C <sub>6</sub> H <sub>5</sub> — <i>p</i> -CH <sub>3</sub> <i>p</i> -Tolyl		A	.5	.05	99	79	62
26	I	—CH <sub>2</sub> C <sub>6</sub> H <sub>5</sub> Benzyl		A	.75 .75 .5	0 .025 .025	82 98 97	43 83 61	42 42 42
27	II				.75 .4	0 .025	72 99	65 71	42 52
28	I	—CH <sub>2</sub> C <sub>6</sub> H <sub>4</sub> — <i>p</i> -Cl <i>p</i> -Chlorobenzyl	(M.P. 70°)	C	1.0 .4	0 .025	(50) 93	46 69	50 53
29	II				1.0 .4	0 .025	(74) 98	66 86	43 58
30	I	—CH <sub>2</sub> C <sub>6</sub> H <sub>4</sub> — <i>o</i> -Cl <i>o</i> -Chlorobenzyl		C	.4 .4	.05 .025	94 92	65 56	42 53
31	I	—CH <sub>2</sub> C <sub>6</sub> H <sub>4</sub> — <i>p</i> -CH(CH <sub>3</sub> ) <sub>2</sub> <i>p</i> - <i>iso</i> -Propylbenzyl		C	1.0	.025	92	75	55
32	I	—CH <sub>2</sub> CH <sub>2</sub> C <sub>6</sub> H <sub>5</sub> 2-Phenylethyl		B	1.0 .4	0 .05	(76) 96	64 79	63 51
33	II				1.0 .4	0 .05	(81) 95	74 79	63 51
34	I	—(CH <sub>2</sub> ) <sub>2</sub> —O—C <sub>2</sub> H <sub>5</sub> 2-Ethoxyethyl	174-175/1	D	1.0 .4	0 .025	95 96	87 79	55 57
35	II		164-167/1		1.0 .4 .2	0 .025 .025	83 99 97	76 87 69	55 57 57

TABLE I—(Continued)

Compound No.	Compound		B. P. °C./mm.	RSH from*	Concn. g. per 100 ml.	Pyrethrins g. per 100 ml.	10-Min. knock- down %	24-Hr. kill %	OTI kill %*
	Type	Wherein R is:							
36	I	$-(CH_2)_2-O-(CH_2)_5CH_3$ 2- <i>n</i> -Butoxyethyl	182-185/1	D	1.0	0	84	80	63
	II				.2 .4	.05 .025	98 97	81 82	50 62
37	I	$-(CH_2)_2-O-(CH_2)_5CH_3$ 2- <i>n</i> -Butoxyethyl	169-172/1	D	1.0	0	(82)	78	63
	II				.2 .4	.05 .025	98 95	90 86	51 62
38	I	$-(CH_2)_2-O-(CH_2)_2-O-(CH_2)_5CH_3$ 2-(2- <i>n</i> -Butoxyethoxy)ethyl	204-207/1	D	1.0	0	96	95	66
	II				.4 .2 0 .2	0 .025 .0125 .025 +0.1 g. DDT .0125 +0.1 g. DDT	51 (74) 97 95 89 96	60 70 70 86 95	55 55 48 48 48
39	I	$-(CH_2)_2-O-(CH_2)_2-O-(CH_2)_5CH_3$ 2-(2- <i>n</i> -Butoxyethoxy)ethyl	194-197/1	D	1.0	0	84	82	66
	II				.4 .4 .2 .1 .1 .15 0 .15	0 .025 .025 .025 .05 .0125 .025 +0.1 g. DDT .0125 +0.1 g. DDT	69 (75) 98 99 99 99 92 82 95	71 90 76 69 81 42 69 87	55 55 55 52 58 40 40 40
40	I	$-(CH_2)_2-O-(CH_2)_2-O-(CH_2)_5CH_3$ 2-(2- <i>n</i> -Hexyloxyethoxy)ethyl		E	1.0	0	73	68	55
	II				.4	.025	95	79	62
41	I	$-(CH_2)_2-O-(CH_2)_2-O-(CH_2)_5CH_3$ 2-(2- <i>n</i> -Hexyloxyethoxy)ethyl		E	1.0	0	83	82	55
	II				.4 .2	.025 .025	96 96	88 69	62 51

TABLE I—(Continued)

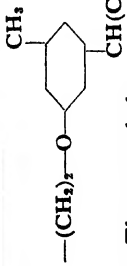
Compound No.	Compound		B. P. °C./mm.	RSH from*	Concn. g. per 100 ml.	Pyrethrins g. per 100 ml.	10-Min. knock-down %	24-Hr. kill %	OTI kill %**
	Type	Wherein R is:							
42	I	$-(CH_2CH_2O)_3-(CH_2)_2CH_3$			1.0	0	92 (97)	92	62
43	II	2-[2-(2- <i>n</i> -Butoxyethoxy)ethoxy]ethyl		E	.6	.025	99	80	54
					1.0	0	94 (97)	96	62
					.4	.025	98	85	62
					.2	.025	98	78	62
44	I	$CH_2-CH_2$			1.0	0	95	93	66
45	II	$-(CH_2)_2O-CH_2CH_2O-CH_2$ 3-Tetrahydrofuryloxypropyl		E	.4	.025	100	92	62
					1.0	0	92	91	66
					.4	.025	99	98	62
					.2	.025	98	64	51
46	II	$CH_2-CH_2$ $-(CH_2)_2O-(CH_2)_2O-CH_2CH_2O-CH_2$ 2-(2-Tetrahydrofuryloxyethoxy)ethyl		E	1.0	0	97	97	55
					.4	.025	98	90	62
					.2	.025	97	64	50
47	I	$-(CH_2)_2O-C_6H_5$			1.0	0	80	77	66
48	II	2-Phenoxyethyl		F	.4	.025	96	78	45
					.2	.025	96	67	48
					1.0	0	80	77	66
					.4	.025	99	93	45
					.2	.025	94	71	48
49	II	$-(CH_2)_2O-C_6H_4-p-CH_3$ 2-( <i>p</i> -Methylphenoxy)ethyl		F	.4	.025	99	85	52
					.2	.025	97	63	52
50	I				1.0	0	68	45	65
51	II	2-Thymoxyethyl		F	1.0	.025	99	73	65
					.6	.025	99	61	62
					1.0	0	59	47	65
					1.0	.025	99	79	65
					.6	.025	97	63	62

TABLE I—(Continued)

Com- pound No.	Compound		B. P. °C./mm.	RSH from*	Concn. g. per 100 ml.	Pyrethrins g. per 100 ml.	10-Min. knock- down %	24-Hr. kill %	OTI kill %**
	Type	Wherein R is:							
52	II	$-(CH_2)_7-O-C_6H_4-p-Cl$ 2-( <i>p</i> -Chlorophenoxy)ethyl		F	1.0 .4 .2	0 .025 .025	73 93 94	72 73 68	55 45 45
53	I	$-(CH_2)_7-O-(CH_2)_7-O-C_6H_5$ 2-(2-Phenoxyethoxy)ethyl			1.0 .4 .2	0 .025 .025	99 97 97	98 87 66	65 50 46
54	II			F	1.0 .4 .2	0 .025 .025	94 96 97	94 90 73	65 50 45
55	I	$-(CH_2)_7-O-CH_2C_6H_5$ 2-Benzoyethyl			1.0 .4 .2	0 .025 .025	90 (95) 98 95	92 71 55	65 43 43
56	II			D	1.0 .4 .2 .1	0 .025 .025 .05	90 (93) 90 98 98 98	91 82 75 60 73	65 41 45 41 45
57	I	$-(CH_2)_7-O-C_6H_4-p-OCH_3$ 3-( <i>p</i> -Methoxyphenoxy)propyl			1.0 .4 .2	0 .025 .025	88 (92) 97 95	88 92 68	62 57 53
58	II			F	1.0 .4 .2	0 .025 .025	78 97 93	76 81 62	62 60 53
59	I	$-(CH_2)_7-O-C_6H_4-o-OCH_3$ 3-( <i>o</i> -Methoxyphenoxy)propyl			.4	.025	96	81	57
60	II			F	.4	.025	96	78	60
61	I	$-(CH_2)_7-O-C_6H_4-m-OCH_3$ 3-( <i>m</i> -Methoxyphenoxy)propyl			.4	.025	97	73	57
62	II			F	.4	.025	95	79	57

TABLE I—(Continued)

Com- pound No.	Compound Wherein R is:	B. P. °C./mm.	RSH from*	Concn. g. per 100 ml.	Pyrethrins g. per 100 ml.	10-Min. knock- down %	24-Hr. kill %	OTI %**
	Type							
63	I —(CH <sub>2</sub> CH <sub>2</sub> -O-) <sub>7</sub> -C <sub>4</sub> H <sub>9</sub> - <i>m</i> -OCH <sub>3</sub> 2-[2-( <i>m</i> -Methoxyphenoxy)ethoxy]ethyl		F	.4	.025	98	84	53
64	II			.4	.025	99	94	53
65	II —C <sub>4</sub> H <sub>9</sub> O Furfuryl	160-170/2 (Crude)	G	.4	.025	97	66	56
66	I —(CH <sub>2</sub> ) <sub>2</sub> -O-(CH <sub>2</sub> ) <sub>2</sub> -S-(C <sub>3</sub> H <sub>7</sub> )			1.0 .4	0 .025	69 98	63 82	46 56
67	II 3,4-CH <sub>2</sub> O <sub>2</sub> C <sub>6</sub> H <sub>3</sub> -(C <sub>3</sub> H <sub>7</sub> )		††	1.0 .4 .2	0 .025 .025	52 (66) 97 95	65 91 70	41 56 46
68	I —CH <sub>2</sub> CH <sub>2</sub> OH 2-Hydroxyethyl	196-199/3	A	1.0	.025	92	35	65
69	II	178-180/3		1.0	.025	91	27	65
70	I —CH <sub>2</sub> CH <sub>2</sub> -O-C(=O)-(CH <sub>2</sub> ) <sub>2</sub> CH <sub>3</sub> 2- <i>n</i> -Butyryloxyethyl		†	2.0	.025	97	35	65

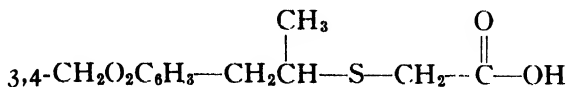
\* See text for the source of the mercaptan (RSH) used in the synthesis of the compound.

\*\* The 10-minute knockdown of the OTI was over 95 per cent in all cases.

† The figures in parentheses indicate the percentage knockdown based upon the number of flies down at the end of the process of picking up the paralyzed flies. This corresponds approximately to 15-minute knockdown.

†† Compounds 66 and 67 are, respectively, the products of HS-(CH<sub>2</sub>)<sub>2</sub>-O-(CH<sub>2</sub>)<sub>2</sub>-SH with 2 molar equivalents of safrole and with 2 molar equivalents of isosafrole.‡ Compound 70 is the product from compound 68 and *n*-butyric anhydride.

*Acid II.* A mixture of 81 g. of isosafrole, 46 g. of mercaptoacetic acid and a few drops of ascaridole was subjected to the same treatment as was used in the preceding synthesis. The yield of the product, which was a viscous oil, was 95 g. A portion of this was distilled at 208°–212° C. at 2 mm. pressure. This product, Acid II, has the probable formula:



*Neutral equivalent.* Calculated for C<sub>12</sub>H<sub>14</sub>O<sub>4</sub>S: 254.3. Found: 260.

*N-Substituted amides.* Acids I and II were each converted into their acid chlorides by treatment with thionyl chloride in benzene solution. The excess thionyl chloride and benzene were removed by distillation under reduced pressure. For the formation of each of the amides listed in Table II, an acid chloride in benzene solution was treated with an excess of the appropriate amine and warmed gently for several hours, or allowed to react at room temperature until the next day. The reaction mixture was then washed with sufficient dilute hydrochloric acid (or dilute sulphuric acid in the case of the dicyclohexylamide) and then washed with sufficient dilute aqueous sodium hydroxide. The N-cyclohexyl and the N-benzyl amides were recrystallized from ethanol, and the other solid amides from a mixture of ethyl ether and low boiling petroleum ether. The liquid amides, with the exception of the morpholides, are quite soluble in "Deo-base;" the N,N-dibutyl and the N,N-diamyl amides are very soluble in this solvent. In preparing fly spray solutions it was necessary in the case of the solid amides and the morpholides to include a co-solvent with the "Deo-base." Ten per cent or less of acetone was sufficient.

*Esters.* The ethyl ester corresponding to Acid II was made by the addition of ethyl mercaptoacetate to isosafrole. The other esters listed at the end of Table II were made by esterification of the acids with the alcohols in benzene solution with the use of *p*-toluenesulphonic acid as catalyst and the removal of water by azeotropic distillation.

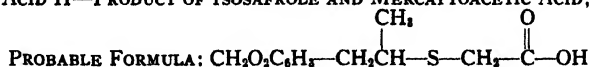
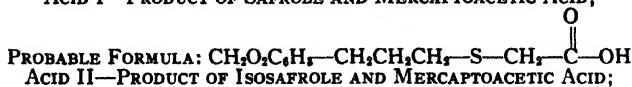
*Toxicity to houseflies of N-substituted amides and esters of Acids I and II.* The data are shown in Table II. All of the amides contribute to the knock-down and kill when tested in admixture with pyrethrins. The amides tested without admixed pyrethrins showed considerable paralyzing and killing ability. The observation made in regard to the previously discussed compounds of Class I, namely, the greater activity of compounds derived from isosafrole, does not appear to hold true for the amides.

Compound 87, the N,N-di-*n*-butylamide of Acid I appears to be the most active amide and its excellent solubility in "Deo-base" also is in its favor. It is even more active than any compound of Class I. The N-cyclohexylamides of both of the acids, compounds 77 and 78, and the piperidides,



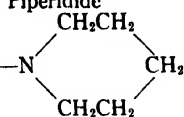
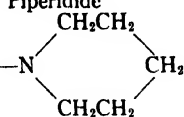
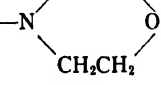
TABLE II

TOXICITY TO HOUSEFLIES OF N-SUBSTITUTED AMIDES AND ESTERS OF THIO ETHER ACIDS  
ACID I—PRODUCT OF SAFROLE AND MERCAPTOACETIC ACID;



Compound No.	Compound		M. P. (uncorr.) ° C.	Concn. g. per 100 ml.	Pyrethrins g. per 100 ml.	10-Min. knock-down %	24-Hr. kill %	OTI kill %**
	Derived from acid	And is thereof the:*						
71	I	N-Ethylamide —NH—CH <sub>2</sub> CH <sub>3</sub>	61	.4	.025	96	58	51
72	I	N-n-Butylamide —NH—(CH <sub>2</sub> ) <sub>3</sub> CH <sub>3</sub>	50	.4 .2	0 .025	72 96	67 55	64 50
73	II		62	.2 .1	.025 .025	99 94	86 70	57 57
74	I	N-iso-Butylamide —NH—CH <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	47	.4 .2	0 .025	90 98	56 77	40 54
75	II		76	.2	.025	96	73	57
76	I	N-Allylamide —NH—CH <sub>2</sub> —CH=CH <sub>2</sub>	74	.4	.025	97	53	51
77	I	N-Cyclohexylamide  —NH—CH <span style="border: 1px solid black; padding: 2px 10px;">(CH<sub>2</sub>)<sub>4</sub></span>	85	.4	0	92	52	39
				.4	.025	99	94	39
				.2	.025	99	83	39
				.1	.025	98	63	44
				.05	.025	97	51	53
				.1	.0125	99	45	46
				0	.025			
					+0.1 g. DDT	86	74	46
				.1	.0125			
					+0.1 g. DDT	97	93	46
78	II		100	.4 .4 .2 .1 .05	0 .025 .025 .025 .025	88 99 98 99 97	38 94 86 80 51	39 39 39 53 53
79	I	N-Benzylamide	82	.4	.025	96	48	51
80	II	—NH—CH <sub>2</sub> C <sub>6</sub> H <sub>5</sub>	85	.4 .2	0 .025	78 96	23 65	35 35
81	I	N,N-Diethylamide	Oil	.2	.025	95	75	50
82	II	—N(—C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub>	Oil	.2	.025	95	59	51
83	I	N,N-Di-n-propylamide	Oil	.2 .1	.025 .025	99 97	71 59	43 43
84	II	—N(—CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub> ) <sub>2</sub>	Oil	.2	.025	97	79	57
85	I	N,N-Di-iso-propylamide	Oil	.2	.025	99	66	43
86	II	$\overset{\text{CH}_3}{\underset{ }{\text{N}}}(\text{—CH—CH}_3)_2$	Oil	.2	.025	96	73	57

TABLE II (Continued)

Compound No.	Compound		M. P. (uncorr.) ° C.	Concn. g. per 100 ml.	Pyrethrins g. per 100 ml.	10-Min. knock-down %	24-Hr. kill %	OTI kill %**
	Derived from	And is thereof the:*						
87	I	N,N-Di-n-Butylamide —N(—CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub> ) <sub>2</sub>	Oil	.4	0	76	57	51
				.4	.025	99	92	51
				.2	.025	98	81	51
				.1	.025	96	65	49
				.05	.025	99	45	40
				.1	.0125	99	45	46
				0	.025			
					+0.1 g. DDT	86	74	46
				.1	.0125			
					+0.1 g. DDT	97	90	46
88	II		Oil	.4	0	65	40	55
				.2	.025	97	65	55
				.1	.025	97	51	55
89	I	N,N-Diamylamide (mixture of isomers)	Oil	.2	.025	98	65	43
		—N(C <sub>5</sub> H <sub>11</sub> ) <sub>2</sub>		.1	.025	96	60	43
90	II		Oil	.2	.025	91	64	55
91	I	N,N-Dicyclohexylamide —N(—C <sub>6</sub> H <sub>11</sub> ) <sub>2</sub>	Oil	.4	.025	98	72	58
92	I	Piperidide 	Oil	.8	0	87	80	53
				.4	0	81	59	50
				.2	.025	100	85	52
				.1	.025	99	67	51
93	II		Oil	.8	0	88	87	53
				.2	.025	99	87	52
				.1	.025	98	55	52
94	I	Morpholide CH <sub>2</sub> CH <sub>2</sub>	Oil	.2	.025	93	53	50
95	II		Oil	.2	.025	96	51	52
96	II	Ethyl ester —O—CH <sub>2</sub> CH <sub>3</sub>	(B.P. 170-171/1)	.5	.025	98	26	57
97	I	n-Butyl ester —O—(CH <sub>2</sub> ) <sub>3</sub> CH <sub>3</sub>	Oil	2.0	.025	99	41	65
98	I	2-Ethylhexyl ester C <sub>2</sub> H <sub>5</sub>	Oil	1.0	.025	86	30	59
99	II	—O—CH <sub>2</sub> —CH—(CH <sub>2</sub> ) <sub>3</sub> CH <sub>3</sub>	Oil	1.0	.025	80	33	52
100	I	Tetrahydrofurfuryl ester	Oil	.5	.025	93	39	51
101	II	—O—C <sub>4</sub> H <sub>7</sub> O	Oil	.5	.025	92	43	51

\* The formula indicates the group which replaces the —OH of the carboxyl group of the acid used to make the compound.

\*\* The 10-minute knockdown of the OTI was over 95 per cent in all cases.

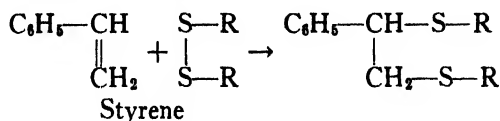
compounds 92 and 93, are also very active. The advantageous use of compounds 77 and 87 as substitutes for part of the pyrethrins in DDT-pyrethrum insecticides is illustrated in a manner similar to that previously discussed in connection with compounds 38 and 39 of Class I.

It is apparent that the amides exhibit synergistic action with pyrethrins in respect to both knockdown and kill.

It appears that esters of Acids I and II that were tested are relatively inactive toward houseflies.

#### CLASS III PRODUCTS—PREPARATION AND TOXICITY TO HOUSEFLIES OF THIO DI-ETHERS

Holmberg (8) found that styrene and certain disulphides when catalyzed by iodine react according to the following equation



The product of styrene and di-*n*-butyl disulphide, compound 102 of Table III, was made by this method and was found relatively inactive toward houseflies.

TABLE III  
TOXICITY TO HOUSEFLIES OF THIO DI-ETHERS

Compound No.	Compound	Boiling point ° C./mm.	Concn. g. per 100 ml.	Pyrethrins g. per 100 ml.	10-Min. knock-down %	24-Hr. kill %	OTI kill %*
102	$\begin{array}{c} \text{C}_6\text{H}_5-\text{CH}-\text{S}-(\text{CH}_2)_3\text{CH}_3 \\   \\ \text{CH}_2-\text{S}-(\text{CH}_2)_3\text{CH}_3 \end{array}$	155-160/1	2.0	.025	98	24	43
103	$\begin{array}{c} 3,4-\text{CH}_2\text{O}_2\text{C}_6\text{H}_3-\text{CH}-\text{S}-(\text{CH}_2)_3\text{CH}_3 \\   \\ \text{CH}-\text{S}-(\text{CH}_2)_3\text{CH}_3 \\   \\ \text{CH}_3 \end{array}$	195-200/1	1.0 .5	0 .025	76 99	55 73	51 51

\* The 10-minute knockdown of the OTI was 99 per cent in these runs.

The analogous product of isosafrole and di-*n*-butyl disulphide, compound 103 of Table III, was made by the same method and was found to be fairly active.

#### CLASS IV PRODUCTS—PREPARATION AND TOXICITY TO HOUSEFLIES OF THIO ETHERS DERIVED FROM OTHER ARYL OLEFINS

The compounds listed in Table IV were prepared in the manner analogous to that used for the preparation of Class I products. The products made with 2-vinylpyridine were purified in the following manner. After termination of the reaction period the reaction mixture was poured into

TABLE IV  
THIO ETHERS DERIVED FROM OTHER ARYL OLEFINS

Compound No.	Probable formula	B. P. ° C./mm.	Product of the peroxide catalyzed addition reaction of:
104	$\text{C}_6\text{H}_5\text{—CH}_2\text{CH}_2\text{—S—CH}_2\text{C}_6\text{H}_5$	190–195/1	Styrene and benzyl mercaptan
105	$p\text{—CH}_3\text{O—C}_6\text{H}_4\text{—CH}_2\overset{\text{CH}_3}{\underset{ }{\text{CH}}}\text{—S—(CH}_2)_7\text{CH}_3$	168–172/1	Anethole and <i>n</i> -octyl mercaptan
106	$3\text{—CH}_3\text{O—4—HO—C}_6\text{H}_3\text{—(CH}_2)_3\text{—S—(CH}_2)_7\text{CH}_3$		Eugenol and <i>n</i> -octyl mercaptan
107	$\text{C}_1\text{H}_4\text{N—CH}_2\text{CH}_2\text{—S—(CH}_2)_3\text{CH}_3$	110–112/2	2-Vinylpyridine and <i>n</i> -butyl mercaptan
108	$\text{C}_6\text{H}_4\text{N—CH}_2\text{CH}_2\text{—S—C}_6\text{H}_4\text{—}p\text{—CH}_3$		2-Vinylpyridine and <i>p</i> -tolyl mercaptan
109	$\text{C}_6\text{H}_4\text{N—CH}_2\text{CH}_2\text{—S—CH}_2\text{C}_6\text{H}_5$		2-Vinylpyridine and benzyl mercaptan
110	$\text{C}_6\text{H}_4\text{N—CH}_2\text{CH}_2\text{—S—(CH}_2)_7\text{CH}_3$	155 157/1	2-Vinylpyridine and <i>n</i> -octyl mercaptan

a great excess of dilute hydrochloric acid; any mercaptan or disulphide present was removed by extraction with ethyl ether. The product was then liberated from its hydrochloride by means of sodium hydroxide and extracted with petroleum ether or ethyl ether. Compound 107 has a very peculiar odor somewhat suggestive of crushed bean leaves.

With the exception of compound 110, none of these compounds contributed appreciably to the kill when solutions containing 2 g. of a compound and 0.025 g. pyrethrins per 100 ml. were tested on houseflies.

TABLE V  
TOXICITY TO HOUSEFLIES OF THE COMPOUND MADE FROM 2-VINYLPYRIDINE AND  
*n*-OCTYL MERCAPTAN  
(COMPOUND 110)

Concn. g. per 100 ml.	Pyrethrins g. per 100 ml.	10-Min. knockdown %	24-Hr. kill %	OTI	
				Kill %	Knock- down %
2.0	0	94	84	57	99
1.0	.05	100	71	58	98
1.0	.025	97	63	56	99

Compound 110, as shown in Table V, is definitely active toward houseflies. The previously discussed data on Class I products would suggest that the use of mercaptans containing ethereal oxygen might lead to more active compounds. This is to be investigated.

**PRELIMINARY TESTS OF VARIOUS SELECTED PRODUCTS AS  
POSSIBLE GENERAL INSECTICIDES**

*Method of testing on certain agricultural insects.* A weighed amount of a compound, usually 0.1 g. in 10 ml. of acetone, was mixed with 90 ml. of a 0.1 per cent aqueous solution of sodium lauryl sulphate and the resulting emulsion was applied to the insect-infested leaves with a hand atomizer. The sprayed specimens were placed in Petri dishes and after allowing a

TABLE VI

**TOXICITY OF SELECTED PRODUCTS OF THE FOUR CLASSES TO CERTAIN AGRICULTURAL  
INSECTS AND MITES**

A 0.1 per cent emulsion was used unless otherwise indicated

Compound		Per cent kill* in 20 hours of insects on plant leaves							
From Table	No.	Bean aphid ( <i>Aphis rumicis</i> L.) agamic, on bush bean		Pea aphid ( <i>Macrosiphum pisi</i> Kltb.) agamic, on horse bean		Red spider mite ( <i>Tetranychus telarius</i> L.) adult and young, on bush bean		<i>Thrips</i> sp. adult and larvae, on chicory	
		No. of insects	Kill %	No. of insects	Kill %	No. of mites	Kill %	No. of insects	Kill %
I	9	216	100			139	85		
	10	146	99						
	13	130	94						
	14	124	79						
	20	45	96						
	24	143	69						
	25	136	69						
	26	158	3			124	100**		
	27	162	9			148	100**		
	28	229	91						
	29	202	82						
	38	96	99	46	99			50	100
	39	101	99	85	88			51	100
	53	92	99	99	97			74	93
	54	46	89	96	78			39	94
	55	47	64	41	78			34	100
	56	67	95	90	100			30	100
II	77	70	99	73	100			44	100
	92	292	99			131	49		
III	102	63	83						
	103	77	42						
IV	104	62	100			68	100**	51	100
	105	111	81						
	106	167	81						
	107	176	100			52	69		
	108	187	97						
	109	211	88			137	100**		

\* In no case was the mortality in a control test greater than 8 per cent.

\*\* A 0.25 per cent emulsion of the chemical was used in these cases.

short time for the evaporation of the acetone, the covers were placed on the dishes. The results were determined after 20 hours.

*Results of tests on certain agricultural insects.* The data are shown in Table VI. Most of the compounds, including those compounds of Classes III and IV which were ineffective against houseflies, were active when tested on the indicated agricultural insects. Compounds 26 and 27, the products from safrole and isosafrole with benzyl mercaptan, appear ineffective toward bean aphids, whereas the related products, compounds 28 and 29, made with *p*-chlorobenzyl mercaptan are relatively effective against this insect.

In contradistinction to the results of the tests on houseflies, the results of the present tests suggest that the methylenedioxy substituent on a benzene ring may not be necessary in the structures of these thio ethers in order to make them toxic to these agricultural insects. A suggestion may be made, however, that the thio ethers containing a methylenedioxy substituent may be expected to be also effective as synergists with pyrethrum not only against flies but against other insects as well. Thus piperine, which also contains the 3,4-methylenedioxyphenyl residue in its structure, and which previously had been shown to be an excellent synergist with pyrethrum as well as a fair toxicant by itself when tested on houseflies (6), was reported by Roark (15) to be a synergist for pyrethrum when tested on a number of agricultural insects although ineffective against these by itself, and piperine was also found by Dewey (2) to be a synergist for pyrethrum when tested on the German cockroach.

*Repellent effect on Mexican bean beetle larvae.* When 0.2 per cent emulsions of each of the following: compounds 9, 10, 26, 39, 47, 54, and 56 of Class I and 87 and 88 of Class II, were similarly tested on Mexican bean beetle (*Epilachna varivestis* Muls.) larvae on bush bean leaves, practically no feeding occurred during the 48-hour test period. Feeding occurred in the control tests.

*Plant injury tests.* Digitalis plants, which appear to be generally very susceptible to injury by chemicals, were tested in the greenhouse. Compounds 9, 38, 39, 53 and 56 of Class I and 83 of Class II, which are among the more active products toward houseflies, were used. Spray emulsions were prepared in the same manner as those used for the tests on agricultural insects. At 0.2 per cent, compound 56 caused no injury during a month, but at 0.4 per cent, it caused moderate leaf injury. At 0.1 per cent, compound 39 caused no injury during the two weeks observed, but at 0.2 per cent, it caused moderate leaf injury. At 0.2 per cent, compounds 9, 38, 53, and 83 each caused some leaf injury within a week.

At 0.1 per cent, compounds 38 and 39 caused no apparent injury to bush beans during two weeks.

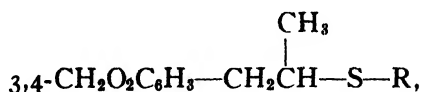
The effects on plants of the other compounds of this paper were not determined. Possibly some of these may be particularly suitable for use on plants.

*Acute toxicity tests on white rats.* Compounds 39 and 56 of Class I and 77 of Class II, which had been found to be among those most active toward houseflies, were each thoroughly incorporated into a separate portion of ground Rockland Rat Diet at a level of 0.2 per cent. One mature white rat was used for each mixture and was fed exclusively on this for a week, after which the rat received the control diet. Neither during the feeding of the test compounds nor during the following month was any harmful effect apparent. The tests suggest that these three compounds and closely related compounds are probably not seriously toxic to warm-blooded animals.

#### SUMMARY

All except two of the 110 compounds investigated were prepared by procedures involving the peroxide catalyzed additions of various sulphhydryl compounds to the olefinic double bonds in safrole and isosafrole, and in a few cases, to the olefinic double bonds in styrene, anethole, eugenol, and 2-vinylpyridine. Two compounds were made by the iodine catalyzed addition of di-*n*-butyl disulphide to the olefinic double bonds of styrene and isosafrole. All of the compounds were tested as insecticides against houseflies by the Peet-Grady method.

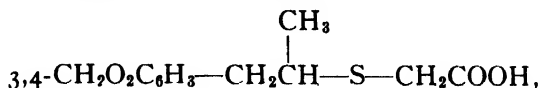
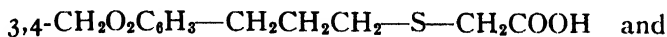
The 34 compounds made from safrole and mercaptans have the probable formula: 3,4-CH<sub>2</sub>O<sub>2</sub>C<sub>6</sub>H<sub>3</sub>—CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>—S—R, and the 36 compounds made from isosafrole and mercaptans have the probable formula:



where R in both cases represents a substituent radical. When the substituent was the same in both types of compounds, the latter was usually more active toward houseflies. The most active compounds of both types were found to be those in which the substituent radical contained one or more ethereal oxygen atoms. Among the most active compounds were those in which the substituent radical was 2-(2-*n*-butoxyethoxy)ethyl, 2-benz-oxyethyl, and 2-(2-phenoxyethoxy)ethyl. The compounds in which the substituent was a simple alkyl, aryl, or aralkyl radical were also active but less so than those having a substituent also containing ethereal oxygen. The active compounds appeared to exert synergistic action with pyrethrum, in respect to both knockdown and kill. Those tested at higher concentrations without admixed pyrethrins also showed considerable paralyzing and killing ability. When the substituent was 2-hydroxyethyl or

the butyric ester of this, the compounds were relatively inactive toward houseflies.

Two acids, having the probable formulae:



were made from mercaptoacetic acid with safrole and isosafrole, respectively. Twenty-five N-substituted amides of these acids were tested on houseflies and were found to be active. These appeared to exert synergistic action with pyrethrum in respect to both knockdown and kill. Those tested at higher concentrations without admixed pyrethrum also showed considerable paralyzing and killing ability. Of the amides tested, the N,N-di-*n*-butylamide of the first acid appears the most promising for use in fly sprays. The N-cyclohexylamides and the piperidides of both acids were also found to be very active. Six esters of these acids were found to be relatively inactive toward houseflies.

The thio di-ether made by the iodine catalyzed addition of di-*n*-butyl disulphide to the olefinic double bond of styrene was relatively inactive toward houseflies, while the analogous compound made from isosafrole was fairly active.

Of seven compounds made from other aryl olefins and mercaptans that were tested, only the compound made from 2-vinylpyridine and *n*-octyl mercaptan was fairly effective toward houseflies.

Preliminary tests of a number of the various thio ethers on agricultural insects and mites such as bean aphids, pea aphids, red spider mites, and a thrips species, showed these compounds, including those thio ethers which did not contain the methylenedioxyphenyl residue and which had been found ineffective toward houseflies, to be toxic to the agricultural insects and mites tested.

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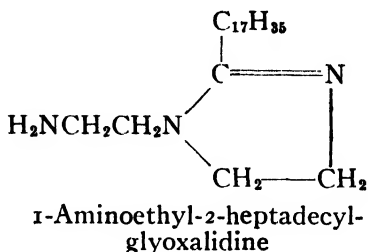
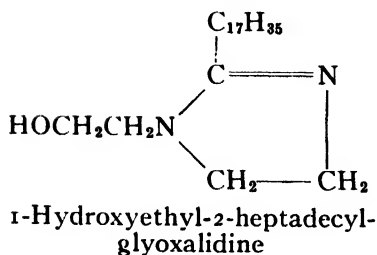
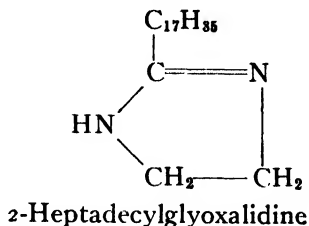
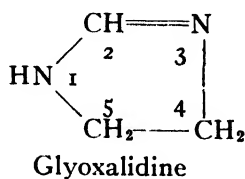


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# GLYOXALIDINE DERIVATIVES AS FOLIAGE FUNGICIDES. I. LABORATORY STUDIES<sup>1,2</sup>

R. H. WELLMAN<sup>3</sup> AND S. E. A. MCCALLAN

In the course of a study of the fungicidal properties of various organic compounds the effectiveness of certain glyoxalidine derivatives was discovered. A large number of derivatives of glyoxalidine<sup>4</sup> were thereafter examined; however, only those which provide pertinent information will be discussed. The glyoxalidine or imidazoline nucleus is shown below together with the three derivatives found to be most active as foliage fungicides. While imidazoline is the preferred chemical designation of this heterocyclic ring, the use of glyoxalidine is also accepted. Since the latter is more familiar to those plant pathologists who have worked with or heard of these materials, the term glyoxalidine will be used throughout.



It is the purpose of this paper to present the results obtained in the laboratory with these materials. The second paper in this series (13) deals with the field performance of certain materials selected on the basis of the

<sup>1</sup> A preliminary report of this work was presented before the New England Division of the American Phytopathological Society, New Haven, Conn., December 6, 1945 (16).

<sup>2</sup> These studies were undertaken by the Crop Protection Institute in cooperation with Boyce Thompson Institute for Plant Research, Inc., under a grant from the Carbide and Carbon Chemicals Corporation.

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<sup>4</sup> These materials were supplied by Dr. G. H. Law of the sponsoring company to whom the authors are also indebted for advice and suggestions during the progress of these investigations.

results reported herein. A foliage fungicide as used here is defined as a material applied to a plant in foliage with the object of controlling disease without phytotoxicity to the host. In interpreting the numerical values presented in the following tables it will be remembered that these are biological data and subject to variation. The precision of the spore-germination method has been extensively discussed (8) and it will be assumed throughout the paper that the reader is cognizant that the values presented are averages of two or more experiments and subject to biological variation.

### FUNGISTATIC ACTION

The slide-germination method (2) was used for the determination of the fungistatic LD<sub>50</sub> values. Essentially this method consists of placing a known concentration of spores in continued contact with a given quantity of chemical. The fungi used were *Sclerotinia fructicola* (Wint.) Rehm., *Glomerella cingulata* (St.) Sp. & von S., *Alternaria solani* (Ell. & Mart.) Jones & Grouet., and *Macrosporium sarcinaeforme* Cav. These fungi are referred to in this paper by their generic names. Table I shows the increase in fungistatic action as the number of carbon atoms in the aliphatic chain in the 2-position is increased to a maximum in the vicinity of 17 carbon atoms beyond which it falls off.

TABLE I  
FUNGISTATIC LD<sub>50</sub> VALUES IN P.P.M. OF GLYOXALIDINE DERIVATIVES

Carbons in 2-position*	Substituent in 1-position	LD <sub>50</sub> values			
		<i>Sclerotinia</i>	<i>Alternaria</i>	<i>Glomerella</i>	<i>Macrosporium</i>
5	Hydroxyethyl	880	1500	3200	1200
11	"	14	20	60	10
13	"	1.1	6.8	11	3.8
15	"	1.0	4.0	4.3	4.1
17	"	0.6	3.6	3.2	3.6
17**	"	2.7	5.7	6.1	6.0
17†	"	1.6	5.6	9.4	8.4
21	"	4.0	11	4.3	21
25	"	116	492	332	229
11	None	83	64	171	56
17	"	1.9	12	15	8.4
17	Aminoethyl	1.4	6.5	5.1	3.9
17	Allyl	1.7	10	5.2	5.9
17	Butyl	2.3	9.2	4.9	7.9
17	Hexyl	4.4	14	3.3	18

\* All members straight chain saturated substituents unless specifically designated.

\*\* Heptadecenyl—unsaturated.

† Heptadecenyl with a methyl group in the 3- or 4-position.

Simple substituents in the 1-position such as hydroxyethyl, aminoethyl, allyl, butyl, or hexyl did not markedly influence the fungistatic activity of these materials. The 2-undecyl and 2-heptadecyl derivatives

were less effective than the hydroxyethyl derivatives perhaps in part because they were much more difficultly dispersed. In the field, however, 2-heptadecylglyoxalidine in a dispersible form was the equal of 1-hydroxyethyl-2-heptadecylglyoxalidine in disease control (13). The methyl group in the 3- or 4-position did not influence fungistatic action.

A logical assumption for this maximum in fungistatic action would be that there is continually increasing toxicity in the series until the point where decreasing solubility allows less than a toxic concentration in solution. It is doubtful that this explanation is tenable in the present case since the 11, 15, 17, and 21 carbon atom members are all soluble to the extent of 5 p.p.m. in water at 20° C. and at 10 p.p.m. the 11 and 15 carbon atom derivatives are soluble while the 17 and 21 carbon atom derivatives are not. An experiment was conducted in which 0.5 g. of activated carbon was added to 50 cc. of 1/100 molar solutions of various substituted glyoxalidines. The samples were agitated for one hour after which the charcoal was removed by filtering and the resulting potency of the glyoxalidine derivative solution measured by the slide-germination technique. The results are presented in Table II from which it will be seen that a greater proportion of chemical was extracted from solutions of the more efficient members, thus indicating that the length of chain might influence efficiency of removal by the spore. However, when the experiment was repeated substituting spores of *Tilletia tritici* (Bjerk.) Wint. for activated carbon, this relationship could not be demonstrated.

TABLE II  
REMOVAL OF GLYOXALIDINE DERIVATIVES FROM SOLUTION BY ACTIVATED CARBON AS MEASURED BY FUNGISTATIC ACTION OF SOLUTIONS AFTER TREATMENT AS COMPARED TO UNTREATED SOLUTIONS. TEST ORGANISM—*SCLEROTINIA FRUCTICOLA*

Carbons in 2-position	LD <sub>50</sub> in p.p.m.		Ratio, treated to non-treated
	Not treated	Treated	
7	2160	12,500	5.8
11	23	700	31
13	26	830	32
15	3.0	11,000	3600
17	1.2	7,400	6200

#### FUNGICIDAL ACTION

An experiment was conducted to determine the fungicidal (1, 6) effectiveness of these materials as contrasted with the fungistatic action. Using the technique described elsewhere (6) it was found that for 1-hydroxyethyl-2-heptadecylglyoxalidine and 2-heptadecylglyoxalidine concentrations 100 times stronger than those necessary for fungistatic LD<sub>50</sub> values did not give fungicidal LD<sub>50</sub> values. It was concluded therefore

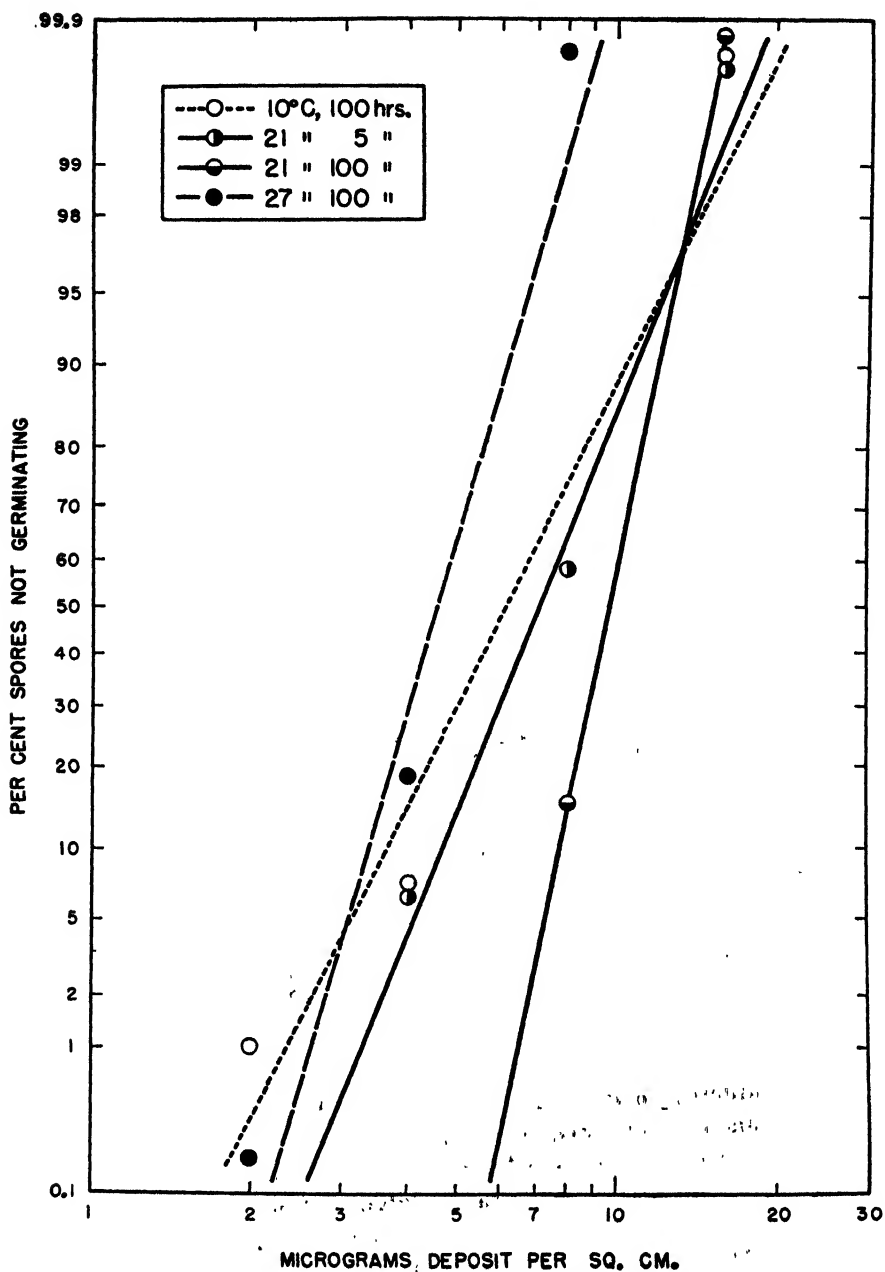


FIGURE 1. Toxicity curves for 1-hydroxyethyl-2-heptadecylglyoxalidine on spores of *Alternaria solani* at different times and temperatures.

that these materials were much more active in inhibiting the germination of spores than in killing the spores.

#### SLOPE OF TOXICITY CURVE; TIME AND TEMPERATURE EFFECTS

Typical toxicity curves obtained by the slide-germination technique are presented in Figure 1. These curves were obtained using spores of *Alternaria solani* and 1-hydroxyethyl-2-heptadecylglyoxalidine. It is evident from this figure that the curves are very steep (9) and that time of counting and temperature (14) have only a minor influence on the slope and position of the curve. Thus at 21° C. and 100 hours, doubling the concentration which prevents only 2 per cent of the spores from germinating will result in preventing 98 per cent from germinating. Further, the concentration necessary to inhibit spore germination is not highly dependent on length of infection period or on temperature. In these respects the glyoxalidine derivatives differ markedly from tetramethylthiuram disulphide as shown by Dimond *et al.* (4) and from copper sulphate and copper zeolite (14). A graphic comparison of the behavior of various fungicides at different temperatures is shown in an earlier paper (14, Figs. 3 and 5) where 1-hydroxyethyl-2-heptadecylglyoxalidine is labelled Organic 337.

#### COMPATABILITY WITH LEAD ARSENATE AND SUMMER OIL

Table III shows the fungistatic compatability of 1-hydroxyethyl-2-heptadecylglyoxalidine with acid lead arsenate and with summer oil<sup>5</sup> as

TABLE III  
LD<sub>50</sub> VALUES IN P.P.M. OF 1-HYDROXYETHYL-2-HEPTADECYLGLYOXALIDINE ALONE AND IN THE PRESENCE OF ACID LEAD ARSENATE AND SUMMER OIL

Concn. of chemicals on basis of 100 gal. of water	<i>Sclerotinia</i>	<i>Alternaria</i>
1 lb. 1-hydroxyethyl-2-heptadecylglyoxalidine	1.9	5.2
1 lb. " + 8 lb. acid lead arsenate	1.0	2.4
1 lb. " + 1 pt. summer oil	1.5	6.6

measured by the slide-germination technique. Mixtures were made which contained the following ratios of ingredients: 1 lb. 1-hydroxyethyl-2-heptadecylglyoxalidine, 8 lb. acid lead arsenate, 100 gal. water; 1 lb. 1-hydroxyethyl-2-heptadecylglyoxalidine, 1 pint summer oil, 100 gal. water. As Table III shows, neither acid lead arsenate nor summer oil in the proportions used adversely affected the fungistatic action. The increased action with acid lead arsenate is presumably due to the fungistatic action of lead arsenate itself.

<sup>5</sup> 75 Saybolt Universal Seconds at 100° F. and an unsulphonatable residue of 99.0 per cent.

## TENACITY ON GLASS SLIDES

The tenacity of the substituted glyoxalidines on glass slides was determined using the method described previously (10). A series of dosages were applied to glass slides by means of the settling tower technique (11) and the slides were then subjected to one inch of laboratory "rain." LD<sub>50</sub> values were then obtained for this set of slides and for a duplicate set which had not been subjected to rain. The ratio of the latter to the former gives the tenacity index (2). The results are presented in Table IV which shows that 2-heptadecylglyoxalidine had some tenacity on glass slides but could not compare with standard laboratory Bordeaux mixture (3) in this

TABLE IV  
TENACITY ON GLASS SLIDES

Compound	LD <sub>50</sub> values mgm./sq.cm.				Tenacity index	
	No rain		1" lab. rain			
	S.f.	A.s.	S.f.	A.s.	S.f.	A.s.
2-Heptadecylglyoxalidine	0.15	0.72	0.49	2.9	0.31	0.40
1-Hydroxyethyl-2-heptadecylglyoxalidine	0.03	0.18	2.6	> 5.2	0.001	< 0.003
1-Aminoethyl-2-heptadecylglyoxalidine	0.02	0.13	3.2	> 2.1	0.006	< 0.006
Bordeaux mixture as Cu	0.16	0.20	0.21	0.21	0.75	0.96

S.f. = *Sclerotinia fructicola*; A.s. = *Alternaria solani*.

respect while 1-hydroxyethyl-2-heptadecylglyoxalidine and 1-aminoethyl-2-heptadecylglyoxalidine were very easily removed from glass slides in this experiment. In view of the field performance of these materials (13) and the results previously obtained by Miller (12) on a group of copper materials, the value of the tenacity test in predicting field performance is seriously questioned.

## PHYTOTOXICITY

Phytotoxicity was determined in the greenhouse by applying a series of concentrations of chemical to bean (*Phaseolus vulgaris* L. var. Bountiful), buckwheat (*Fagopyrum esculentum* Gaertn.), and tobacco (*Nicotiana tabacum* L.), according to the procedure previously outlined (15).

The results obtained in a series of phytotoxicity experiments are presented in Table V. It will be seen that with a saturated side-chain in the 2-position, phytotoxicity increases rapidly with chain length until the chain length reaches 11 carbon atoms and then drops off rapidly as the chain length extends beyond 15 carbon atoms. The substitution of an unsaturated 17 carbon atom chain (heptadecenyl) for a saturated 17 carbon atom chain markedly increases phytotoxicity and the quaternary diethyl

sulphate addition compound is still more phytotoxic. The substituent in the 1-position has a marked effect on phytotoxicity as compared to its minor role in fungistatic action. Thus introduction of the allyl, butyl, or hexyl groups in the 1-position greatly increases phytotoxicity. The 2-heptadecylglyoxalidines thus far discovered which are the least phytotoxic are the ones which have either the aminoethyl or hydroxyethyl group in the 1-position or are unsubstituted in this position.

Figure 2 shows graphically the relationship between fungistatic action and phytotoxicity for a series of 1-hydroxyethylglyoxalidines. The values

TABLE V  
THRESHOLD OF PHYTOTOXICITY IN PER CENT SPRAY CONCENTRATION OF CERTAIN  
GLYOXALIDINE DERIVATIVES

Carbons in 2-position*	Substituent in 1-position	Tobacco	Buckwheat	Bean
5	Hydroxyethyl	0.71	0.50	0.35
7**	"	0.50	0.25	0.25
11	"	0.04	0.02	0.04
15	"	0.06	0.06	0.04
17	"	0.50	0.50	0.35
25	"	1.4	1.4	1.4
17†	"	0.09	0.07	0.10
17††	"	0.04	0.04	0.03
11	None	0.03	0.03	0.03
17	"	0.35	0.35	0.35
17	Aminoethyl	> 1.00	> 1.00	> 1.00
17	Allyl	0.03	0.02	0.02
17	Butyl	0.09	0.04	0.03
17	Hexyl	0.06	0.06	0.04

\* All members straight chain saturated substituents unless specifically designated.

\*\* Branched chain (1-ethylamyl).

† Unsaturated (heptadecenyl).

†† Quaternary diethyl sulphate addition compound of heptadecenyl derivative.

given for threshold of phytotoxicity are averages obtained for tobacco, buckwheat, and bush bean, while those for fungistatic LD<sub>50</sub> are averages for *S. fructicola*, *A. solani*, *G. cingulata*, and *M. sarcinaeforme*. From Figure 2 the ratio of threshold of phytotoxicity concentration to fungistatic LD<sub>50</sub> is 13.5 for 1-hydroxyethyl-2-undecylglyoxalidine while for 1-hydroxyethyl-2-heptadecylglyoxalidine this ratio is 1450. This ratio of effective to phytotoxic concentration may be different in the field but it is indicated that there is a greater margin of safety with the 17 carbon atom derivative than with the 11 carbon atom derivative.

#### CONTROL OF DISEASE IN GREENHOUSE

In greenhouse experiments with the tomato foliage diseases, late and early blights (7), the heptadecylglyoxalidines did not control disease at concentrations which were injurious to tomato foliage. Thus 2-heptadecyl-



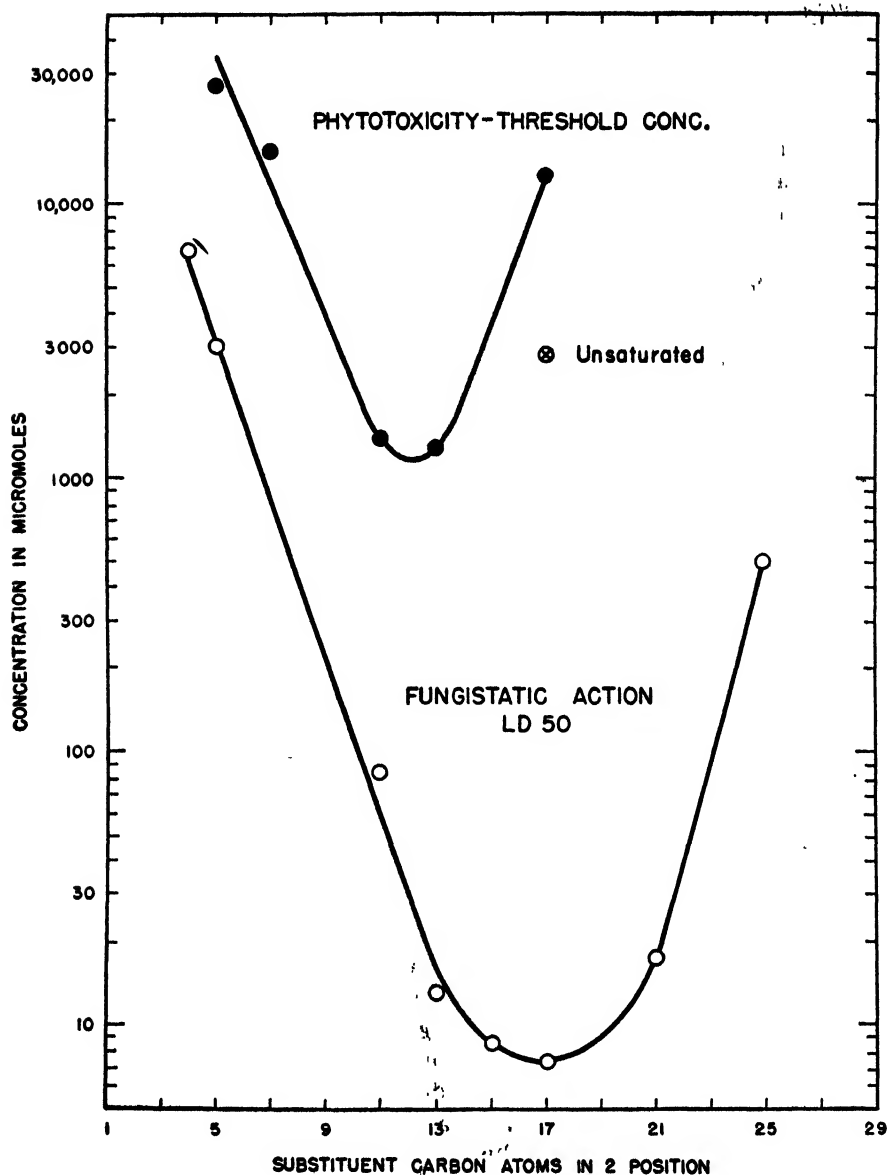


FIGURE 2. Relationship between fungistatic action and phytotoxicity for a series of 1-hydroxyethylglyoxalidines. Note maximum spread between two curves at 17 substituent carbon atoms in 2-position.

glyoxalidine gave a D rating<sup>6</sup> for late blight control and an E rating on early blight when applied at 0.2 per cent concentration. At this concentration it gave a C phytotoxic rating. The other two most promising derivatives, 1-hydroxyethyl-2-heptadecyl- and 1-aminoethyl-2-heptadecyl-, gave somewhat better control and slightly less phytotoxicity; however, neither could be considered satisfactory on tomatoes in view of the performance of standard materials (7).

The glyoxalidine derivatives tested for tomato foliage diseases were also tested on snapdragon rust (5). Using 0.2 per cent sprays, good control was obtained without phytotoxic effects; thus 1-aminoethyl-2-heptadecylglyoxalidine allowed only 2 per cent disease to develop. At 0.04 per cent concentration of spray, however, this material allowed 40 per cent rust to develop. In view of the LD<sub>95</sub> of 0.0007 per cent for Fermate (70 per cent ferric dimethyldithiocarbamate) on this disease (5), their performance on the rusts must be considered mediocre.

These greenhouse findings are borne out in field experimentation (13) where the substituted glyoxalidines were unsatisfactory for control of late blight on potato and only moderately satisfactory for control of cedar-apple rust. However, certain derivatives of the glyoxalidines proved to be very satisfactory against apple scab and cherry leaf spot.

In preliminary tests where they were used as dips in control of wheat smut (bunt) those tested on tomato foliage diseases gave perfect control of smut when used at concentrations of 0.1 per cent but not at 0.01 per cent. This work was not pursued further because seed treatment is much more easily accomplished by dusts and the physical state of these materials makes preparation of a dust somewhat difficult.

#### SUMMARY

1. Results indicate the promise of 2-heptadecyl-, 1-hydroxyethyl-2-heptadecyl-, and 1-aminoethyl-2-heptadecylglyoxalidine or imidazoline as foliage fungicides since they combine high fungistatic action with low phytotoxicity.

2. In slide-germination tests, maximum fungistatic action is achieved with glyoxalidine derivatives having a straight chain substituent containing 13 to 17 carbon atoms in the 2-position. Addition of acid lead arsenate or summer oil in proportions used in the field does not interfere with fungistatic action.

3. Maximum phytotoxicity in the greenhouse is reached with the 11 to 13 carbon atom derivatives. The ratio of highest concentration giving no plant injury to LD<sub>50</sub> value for 1-hydroxyethyl-2-undecylglyoxalidine is 13.5 and for 1-hydroxyethyl-2-heptadecylglyoxalidine 1450. Side-chain un-

<sup>6</sup> For explanation of ratings see (15). An A rating is considered good for disease control or non-phytotoxicity while an E rating represents no control or extreme phytotoxicity.

saturation increases phytotoxicity as does increasing length of chain in the 1-position.

4. In the greenhouse, these materials were phytotoxic to tomatoes at concentrations which would not control late blight and were moderately effective against snapdragon rust without phytotoxicity.

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## GLYOXALIDINE DERIVATIVES AS FOLIAGE FUNGICIDES. II. FIELD STUDIES<sup>1,2,3</sup>

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While it is perhaps superfluous to call attention to the recent great interest in the possibilities of new organic fungicides as foliage sprays, such new materials should receive adequate and thorough field testing before they appear on the market. The following account of field studies on the glyoxalidine derivatives is offered as a contribution to that end.

The glyoxalidine derivatives were found to show possibilities as fungicides in laboratory tests described in the first paper of this series (6). From among the substituted glyoxalidines so studied, three were tested in the field. These compounds were 1-hydroxyethyl-2-heptadecylglyoxalidine, 2-heptadecylglyoxalidine, and 1-aminoethyl-2-heptadecylglyoxalidine which were identified as C. P. I. compound numbers 337, 341, and 630 respectively. The field tests were initiated in 1941 on the farm of the Pennsylvania State College at State College, Pennsylvania.

### TESTS ON ROSES

In 1941, 1-hydroxyethyl-2-heptadecylglyoxalidine and 2-heptadecylglyoxalidine were tested on roses [*Rosa* sp.], variety World's Fair, for the control of black spot [*Diplocarpon rosae* (Fr.) Wolf]. Ten applications of spray were made during the season, using a compressed air hand sprayer, on single plants randomized in each of nine replicate blocks. The results are given in Table I where it can be seen that the glyoxalidine derivatives gave fair control of black spot on this variety. At 3 lb. per 100 gallons their control was not significantly below that obtained with 2-3-100 Bordeaux which required 2 oz. of Orvus<sup>5</sup> as wetting agent. 2-Heptadecylgly-

<sup>1</sup> A preliminary report of this work was presented before the New England Division of the American Phytopathological Society, New Haven, Conn., December 6, 1945 (5).

<sup>2</sup> These studies were supported in part by the experiment stations represented and in part by a Carbide and Carbon Company fellowship through the Crop Protection Institute.

<sup>3</sup> Authorized for publication on January 8, 1946 as paper No. 1303 in the Journal Series of the Pennsylvania Agric. Exp. Sta. Contributions from the Department of Botany No. 152. Also Paper No. 130 from the Section of Plant Pathology, Biology Department, Virginia Agric. Exp. Sta. Published also with the approval of the Director, West Virginia Agric. Exp. Sta. as Scientific Paper No. 351.

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<sup>5</sup> Sodium lauryl sulphate.

TABLE I  
CONTROL OF BLACK SPOT OF ROSE, VAR. WORLD'S FAIR, 1941

Treatment	Black spotted leaflets per plant	Av. wt. of blossoms per plant, g.
Check (no spray)	352	157
1-Hydroxyethyl-2-heptadecylglyoxalidine 1-100	179	152
1-Hydroxyethyl-2-heptadecylglyoxalidine 3-100	76	89
2-Heptadecylglyoxalidine 3-100	38	158
Bordeaux 2-3-100 + 2 oz. Orvus	24	132
Least sign. diff. 19:1	62	38

oxalidine was better in that it was not injurious at 3 lb. per 100 gallons, while 1-hydroxyethyl-2-heptadecylglyoxalidine at the same strength reduced the weight of the blossoms. However, the dry deposit of these sprays was considerably less noticeable than that of Bordeaux, a very desirable feature in ornamental sprays.

#### TESTS ON APPLES

##### APPLE SCAB CONTROL

In 1941 two applications of 1-hydroxyethyl-2-heptadecylglyoxalidine and 2-heptadecylglyoxalidine at concentrations of 1 lb. per 100 gallons were made on McIntosh and Stayman apple [*Pyrus malus* L.] trees following early season sprays of lime sulphur. Since the new fungicides were not applied before the "cover sprays," no adequate data on scab control were obtained. The general appearance of the foliage, which showed a complete lack of injury, together with the attractive color and finish of the fruit, indicated considerable promise for these materials.

Beginning in 1942, more extensive tests were undertaken with the substituted glyoxalidines as sprays for the control of apple scab [*Venturia inaequalis* (Cke.) Wint.]. Each treatment was replicated four times on randomized single-tree plots on each of the varieties McIntosh and Stayman. The full schedule of late-delayed dormant through cover sprays was

TABLE II  
APPLE SCAB CONTROL 1942

Treatment	Per cent scabby fruit at harvest	
	Stayman	McIntosh
Check (unsprayed)	100.0	99.6
2-Heptadecylglyoxalidine 1.5-100	2.1	1.7
2-Heptadecylglyoxalidine 0.75-100	22.6	27.9
1-Aminoethyl-2-heptadecylglyoxalidine 3-100	1.6	1.7
1-Aminoethyl-2-heptadecylglyoxalidine 1-100	18.4	40.8
Lime sulphur 1-75	2.1	3.0

followed using commercial orchard equipment. The results at harvest given in Table II showed control of scab on the fruit by 2-heptadecylglyoxalidine at 1.5 lb. per 100 gallons and 1-aminoethyl-2-heptadecylglyoxalidine at 3 lb. per 100 gallons to be fully equal to that afforded by the standard lime sulphur sprays.

In 1943 a similar experiment compared 1-hydroxyethyl-2-heptadecylglyoxalidine with standard lime sulphur as well as with Fermate (70 per cent ferric dimethyldithiocarbamate) which at that time was coming into prominence as the outstanding organic fungicide for use on fruit. The results in Table III indicate that 1-hydroxyethyl-2-heptadecylglyoxalidine at 1 lb. per 100 gallons was again equal to lime sulphur and superior to Fermate in controlling scab on the leaves and fruit of the two varieties

TABLE III  
APPLE SCAB CONTROL 1943

Treatment	Per cent scab			
	Stayman		McIntosh	
	Leaves	Fruit	Leaves	Fruit
Check (unsprayed)	82.5	78.3	86.5	99.0
Fermate 0.33-100	51.0	19.5	75.0	66.6
" 1-100	38.5	10.1	47.0	24.5
" 3-100	25.3	2.9	39.2	13.0
1-Hydroxyethyl-2-heptadecylglyoxalidine 0.33-100	41.0	17.0	51.8	68.2
" 1-100	23.4	2.1	23.8	3.0
" 3-100	5.8	0.0	7.8	4.2
Lime sulphur 1-75	15.0	6.1	35.0	12.6

tested. The data of Tables II and III are also shown in Figure 1 where the percentage apple scab on fruit is plotted against the concentration of the fungicide using a logarithmic probability scale. It will be noted that the three glyoxalidine derivatives have essentially similar slopes which are significantly steeper than that of Fermate. Also at the 95 per cent control level, it was estimated that on Stayman in 1943 (Fig. 1 B) only 0.65 lb. of 1-hydroxyethyl-2-heptadecylglyoxalidine would have been required as compared to 2.0 lb. of Fermate.

Since it was noted in 1943 that at the strongest concentration, 3 lb. per 100 gallons, 1-hydroxyethyl-2-heptadecylglyoxalidine produced some foliage injury, it was decided to continue the apple tests in 1944 and 1945 with 2-heptadecylglyoxalidine only. Alternate rows in the orchard were sprayed with standard lime sulphur and with 2-heptadecylglyoxalidine at 1 lb. per 100 gallons. A high-pressure commercial sprayer equipped with a 10 foot tower and 8 nozzle broom was used in 1944; in 1945 the sprays were applied with a "Speed Sprayer." Again, a complete spray schedule was followed, late-delayed dormant through cover sprays, and lead arsenate and

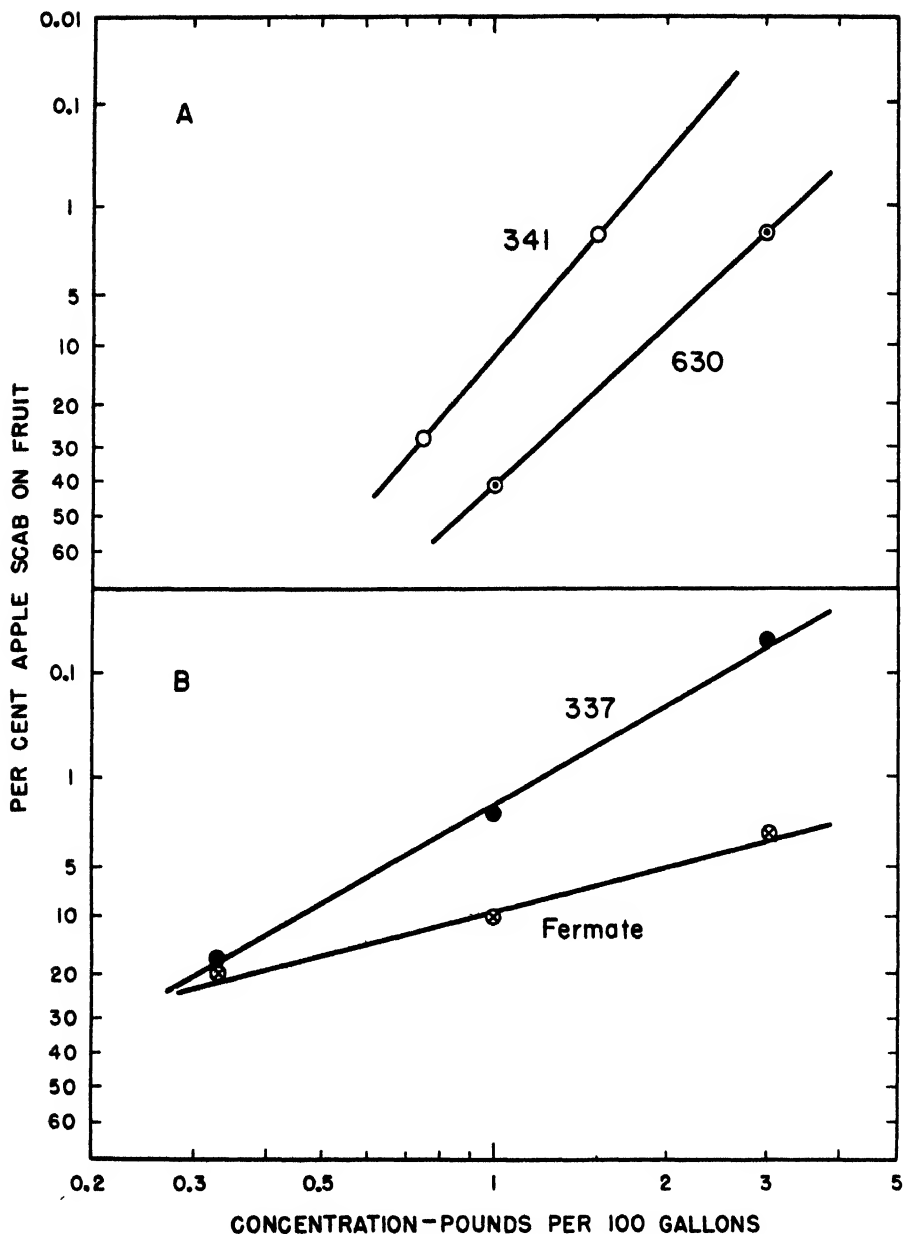


FIGURE 1. Dosage response curves for apple scab control. A. Comparison of 2-heptadecylglyoxalidine (341) and 1-aminoethyl-2-heptadecylglyoxalidine (630) on var. McIntosh in 1942 when there was 3.0% scabby fruit with lime-sulphur 1-75. B. Comparison of 1-hydroxyethyl-2-heptadecylglyoxalidine (337) and Fermate on var. Stayman in 1943 when there was 6.1% scabby fruit with lime-sulphur 1-75.

nicotine sulphate insecticides were included when conditions indicated. The results of these tests appear in Table IV where it is clearly demonstrated that 2-heptadecylglyoxalidine at 1 lb. per 100 gallons effected control of apple scab equivalent to the standard lime sulphur.

Earlier observation had indicated that where these substituted glyoxalidines at safe dosages had been used, the foliage appeared to be much healthier than that sprayed with lime sulphur. An attempt to measure this difference quantitatively by determining the average leaf area per spur utilized the photoelectric apparatus of Frear (1) with minor modifications. Three such sets of measurements revealed a consistent difference in favor of 2-heptadecylglyoxalidine, as seen in Table IV.

TABLE IV  
2-HEPTADECYLGLYOXALIDINE VS. LIME SULPHUR ON APPLE  
A. Variety McIntosh

Treatment	1944		1945 Per cent scabby leaves		
	Per cent scabby leaves	Leaf area per spur	Spurs May 21	Spurs Aug. 9	Terminals Aug. 9
Check (unsprayed)	85.0		63.2		
2-Heptadecylglyoxalidine 1-100	21.6	15.3	4.8	7.4**	30.0**
Lime sulphur 1-75	38.0	13.5	3.4	16.4	48.9

B. Variety Stayman

	Per cent scabby leaves	Leaf area per spur	Per cent scabby leaves	Leaf area per spur
Check (unsprayed)	86.0		59.5	
2-Heptadecylglyoxalidine 1-100	22.2	14.3**	5.8	12.5
Lime sulphur 1-75	23.8	12.5	2.8	11.5

\*\* Highly significant differences.

The 1945 data in Table IV deserve special comment. Since there was no fruit due to heavy frosts during full bloom, only five applications of the sprays were made, the last being the "first cover" on May 14. Three months later, on August 9, scab counts on both spur leaves and terminal leaves showed some rather surprising differences between trees sprayed with 2-heptadecylglyoxalidine and those sprayed with standard lime sulphur as far as the residual effect of the spray was concerned. Spur leaves showed a five-fold increase in scab from the tree average where lime sulphur had been used, and only a two-fold increase following 2-heptadecylglyoxalidine. Terminal leaves, which had largely grown after the last spray and theoretically had little or no protection at all, were much scab-



bier than the spur leaves as was expected, but the number of scabby terminal leaves from lime sulphur sprayed trees was significantly higher than from trees sprayed with 2-heptadecylglyoxalidine. Possibly this is a reflection of redistribution of the fungicides and the less rapid weathering of the 2-heptadecylglyoxalidine.

The 2-heptadecylglyoxalidine is not only compatible physically and biologically with acid lead arsenate, nicotine sulphate, and excess hydrated lime, but Groves (2) has also shown that it is compatible with summer oil on apples both in a complete spray mixture and where oil is applied to the weathered residue.

#### CEDAR-APPLE RUST CONTROL

In 1944, 2-heptadecylglyoxalidine was used in Adams County, Pennsylvania, on apple trees, var. Rome, and in the Shenandoah Valley of

TABLE V  
CONTROL OF CEDAR-APPLE RUST ON APPLE, VARS. ROME AND YORK, IN 1944.  
SPRAYS: PINK, PETAL FALL, FIRST COVER

Treatment	No. rust spots per 100 terminal leaves		Per cent ruste fruit
	Pennsylvania var. Rome	Virginia var. York	Pennsylvania var. Rome
Check (unsprayed)	72.5	522.5	11.3
Fermate 1-100	5.3	21.7	0.9
2-Heptadecylglyoxalidine 1-100	31.2	73.5	5.6
Lime sulphur 1-75	22.3	49.1	5.7
Least sign. diff. 19:1	10.0	30.0	2.2

Virginia on var. York where data on the control of cedar-apple rust [*Gymnosporangium juniperi-virginianae* Schw.] were obtained, as shown in Table V. 2-Heptadecylglyoxalidine was significantly better in control than no spray, was equal to standard lime sulphur, but was significantly poorer than Fermate.

TABLE VI  
POTATO TESTS 1943

Treatment	Yield, bu. per acre	Late blight score*
1-Hydroxyethyl-2-heptadecylglyoxalidine 1-100	160	43
1-Hydroxyethyl-2-heptadecylglyoxalidine 2-100	137	47
2-Heptadecylglyoxalidine 2-100	175	41
Fermate 2-100	183	27
Dithane 2-100	221	20
Bordeaux 8-8-100	226	2

\* Arbitrary scale derived from summation of visual observation of blight severity with high number indicating greater severity.

## TESTS ON POTATOES

During the five-year period covered by these tests, the glyoxalidine derivatives were used on potatoes [*Solanum tuberosum* L.], var. Russet Rural, only in 1943. Laboratory tests on late blight control indicated that these fungicides were not suitable for use on potatoes; the field experiment confirmed this. As is indicated in Table VI, they failed to control adequately late blight [*Phytophthora infestans* (Mont.) de Bary] and did not produce satisfactory yields; in addition, there was positive indication of foliage injury at the concentrations used.

TABLE VII  
DEFOLIATION AND LEAF SPOT CONTROL ON SOUR CHERRY, VAR. MONTMORENCY,  
1942 TO 1945

Pennsylvania								
Treatment	Per cent leaves remaining				Per cent disease-free			
	Sept.	Oct.	Oct.	Oct.	Sept.	Oct.	Oct.	Oct.
	I 1942*	I 1943	I 1944	I 1945	I 1942*	I 1943	I 1944	I 1945
Check	0.1	35.1	3.5	0.0	0.0	9.4	0.0	0.0
Bordeaux**	68.8	63.3	70.2	45.3	80.2	63.8	100.0	9.3
Lime sulphur 2-100	3.9	77.2	69.7	0.0	0.0	54.8	30.2	0.0
Fermate 1-100† (ferric dimethyl- dithiocarbamate)	39.3	78.9	—	—	19.3	49.5	—	—
2-Heptadecylglyoxalidine 1-100	86.7	86.2	96.0	95.4	97.0	75.2	95.6	21.1
Dithane (disodium ethylene bis- dithiocarbamate) 1-100	—	—	25.0	20.7	—	—	0.0	0.0
Phygon 1-100 (dichloronaphtho- quinone)	—	—	91.3	31.6	—	—	60.1	0.5
Virginia								
Check	0.0†	24.6	4.0	0.0	—	0.0	0.0	0.0
Bordeaux**	83.9	72.6	93.8	0.6	—	90.6	90.6	0.0
Lime sulphur 2-100	1.7	56.7	68.5	0.0	—	22.2	1.9	0.0
Fermate 1-100†	33.3	64.0	—	—	—	37.6	—	—
2-Heptadecylglyoxalidine 1-100	88.4	90.1	94.9	21.4	—	91.0	97.8	0.0
Dithane 1-100	—	—	35.2	0.0	—	—	3.1	0.0
Phygon 1-100	—	—	82.1	2.5	—	—	37.0	0.0
West Virginia								
Check	0.0	46.8	—	—	0.0	—	—	—
Bordeaux**	79.9	50.2	—	—	72.3	—	—	—
Lime sulphur 2-100	0.1	59.9	—	—	0.0	—	—	—
Fermate 1-100†	18.3	61.9	—	—	3.3	—	—	—
2-Heptadecylglyoxalidine 1-100	96.1	88.4	—	—	97.3	—	—	—
Dithane 1-100	—	—	—	—	—	—	—	—
Phygon 1-100	—	67.7	—	—	—	—	—	—

\* Courtesy of Dr. H. J. Miller.

\*\* In 1942 and 1943 the Bordeaux formula was 2-8-100; in 1944 1.5-6-100; in 1945 1.5-4-100.

† In 1943 Fermate 1.5-100.

‡ In 1942, all counts October 1.



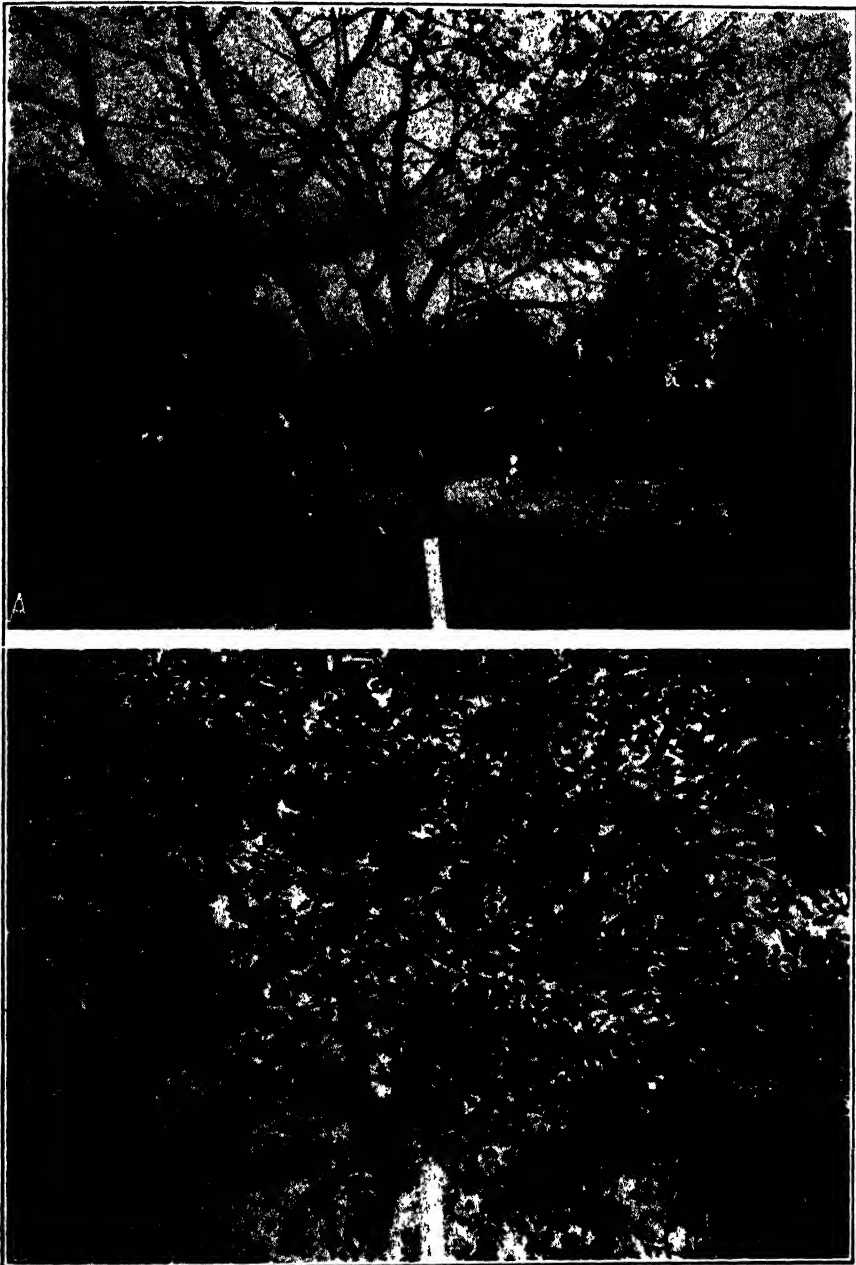


FIGURE 2. Typical comparison of cherry trees sprayed with (A) standard lime sulphur, and (B) 2-heptadecylglyoxalidine to control defoliation by leaf spot. Virginia, September 19, 1942.

## TESTS ON CHERRIES

2-Heptadecylglyoxalidine has been used for four years in experimental spraying of sour cherries [*Prunus cerasus* L.], var. Montmorency. The first year's results (1942) in three states have been published (3) and some of the later work in Pennsylvania and Virginia is mentioned by Lewis and Groves (4) who stated that compound 341 (2-heptadecylglyoxalidine) is very effective in leaf spot control, causes little or no leaf injury, and does not dwarf the fruit. Four years' tests in Pennsylvania and Virginia and two years' tests in West Virginia are summarized in Tables VII and VIII which show 2-heptadecylglyoxalidine to be superior to all other fungicides tested in the control of defoliation caused by the leaf spot fungus [*Coccomyces hiemalis* Higgins] including various strengths of Bordeaux mixture and standard lime sulphur. This latter often fails to give adequate protection but eliminates the severe copper burn which accompanies the use of Bordeaux in wet seasons, hence has been commonly used. A mid-September comparison of the protective efficiency of standard lime sulphur and 2-heptadecylglyoxalidine in preventing defoliation is shown in Figure 2. The color of the fruit is a slightly lighter shade of red and the solids content is apt to be low when 2-heptadecylglyoxalidine is used in sprays immediately preceding harvest.

## SUMMARY

1. Three glyoxalidine derivatives, 1-hydroxyethyl-2-heptadecylglyoxalidine (No. 337), 2-heptadecylglyoxalidine (No. 341), and 1-aminoethyl-2-heptadecylglyoxalidine (No. 630), investigated in the laboratory have been tested in the field during several years for the control of black spot of rose, apple scab and rust, late blight of potato, and cherry leaf spot.

2. On roses (one year's test) 2-heptadecylglyoxalidine at 3 lb. per 100 gallons gave black spot control equivalent to Bordeaux mixture with wetting agent, and with less conspicuous deposit. 1-Hydroxyethyl-2-heptadecylglyoxalidine was less effective and somewhat injurious.

3. In tests over a five-year period in Pennsylvania on apples, var. McIntosh and Stayman, 1-hydroxyethyl-2-heptadecylglyoxalidine and 2-heptadecylglyoxalidine at 1 lb. and 1-aminoethyl-2-heptadecylglyoxalidine at 3 lb. per 100 gallons gave control of apple scab equivalent to standard lime sulphur and much better than Fermate at 3 lbs. 1-Hydroxyethyl-2-heptadecylglyoxalidine caused some foliage injury at 3 lb. per 100 gallons. 2-Heptadecylglyoxalidine at 1 lb. per 100 gallons produced consistently better appearing foliage with greater area per fruit spur than standard lime sulphur and had a marked residual effect on control of scab on foliage. The glyoxalidine derivatives have much steeper dosage response

slopes than Fermate in the field. Against cedar-apple rust 2-heptadecylglyoxalidine was fairly effective but not equal to Fermate.

4. 2-Heptadecylglyoxalidine has been shown to be compatible with acid lead arsenate, nicotine sulphate, excess hydrated lime, and summer oil.

5. The glyoxalidine derivatives caused foliage injury of potato and did not control late blight; this was in accordance with laboratory results.

6. In four years' tests in Pennsylvania and Virginia and two years' tests in West Virginia, 2-heptadecylglyoxalidine on sour cherries, var. Montmorency, at 1 lb. per 100 gallons was demonstrated to be the most effective compound tested for the control of leaf spot defoliation. There was little or no leaf injury and no dwarfing of fruit.

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# INTERNAL PRESSURE NECESSARY TO BREAK SHELLS OF NUTS AND THE ROLE OF THE SHELLS IN DELAYED GERMINATION

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ELTORA M. SCHROEDER

## INTRODUCTION

As will be seen from the following review of the literature, the internal pressure necessary to break fruit or seed coats has been either estimated indirectly or measured semi-directly by determining the tensile strength of fragments of the coats. This research is an attempt to determine directly the internal pressure necessary to rupture certain intact fruit coats after the fruits have been exposed to a variety of environmental conditions. Walnuts, hickory nuts, and butternuts were selected as the fruits to be tested because of the thickness and rigidity of the coats. A hole was drilled through the shell and the hole later threaded with a tapering male tap. A hollow tap with tapering threads was later screwed firmly into the hole and water and air pressure gradually increased until the coats broke.

The amount of internal pressure required to break the shells of walnuts, butternuts, and hickory nuts is several times that estimated previously for breaking the shells of hazelnuts and *Pinus pinea*. The pressure that breaks coats in germination is growth pressure of the embryo and in some cases the endosperm is involved. The growth pressure is, in the main, derived from osmotic pressure of the living tissue. It is possible that swelling pressure of colloids may also play a part in certain cases.

## REVIEW OF LITERATURE

Müller (14) measured both the pressure exerted by the growing embryo and endosperm tissue in the early stages of germination and the pressure necessary to rupture seed and fruit coats. The Pfeffer gypsum block method for measuring growth pressure of various plant organs was modified and adapted to measuring the growth pressure of seed organs. Special adaptation had to be made in the Pfeffer method to provide the seed organs adequate oxygen and water. Also some other changes in the method were found desirable. The following growth pressures were found: *Corylus avellana* embryos 3.305, *Ricinus communis* endosperm 3.104, and *Pinus pinea* endosperm 3.678 atmospheres. Müller mentions that these values are low as compared with the pressures found by Pfeffer for growing plant organs: roots, longitudinal pressures 7 to 12, cross pressures 4.5 to 6 atmospheres; stems, 5.5 to 7.5 atmospheres; and geotropically responding grass nodes, 15 to 16 atmospheres.

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Müller mentions that in determining the pressure necessary to break seed or fruit coats, he had thought of applying gas or water pressure to the interior of intact seed or fruit, but that this was given up because of the technical difficulties involved. Instead, he determined the tensile breaking strength of strips of *Ricinus* coats and rings of *Corylus* and *Pinus* shells. In *Pinus* and *Corylus* shells he attached metal plates inside the rings with hard wax so as to distribute the pull over the whole inner surface of the ring except the dehiscent layers. From the tensile breaking strength thus determined he calculated the internal pressure necessary to break the intact coats. It took less pull to break the coats when the pull was applied for a long period than when applied for a short period. The average breaking strength of the dry *Corylus* shell was more than double the average growth pressure of the growing embryo but the breaking strength of the shell was greatly reduced by imbibition of water. This weakening was sufficient on the average to bring the breaking strength of the shell a little below the growing pressure of the embryo in *Corylus*. The breaking strength of *Pinus* shell fell similarly with imbibition. In both cases the original strength of the shells returned when the coat was again dried. The shell of the black walnut was not weakened by long storage in moist soil or by weak acids. Apparently neither soil organisms nor enzymes nor other materials from the embryo weakened the coats. *Pinus* shell, on the other hand, was weakened at the dehiscent layer by soil organisms and weak acids though the action was slow. Müller mentions that seed and fruit coats with and without dehiscent layers often show cracks after a period in the natural seed bed and before the embryo or endosperm shows any growth. This he attributes to strains set up by repeated moisture absorption and drying.

Crocker (3, p. 107) states "In the light of Müller's recent work it is not strange that some seeds are held in a dormant state because the force of the expanding contents is not sufficient to rupture the coats." On the basis of the length of the isolated embryo of *Alisma* in 2 mol. solution of cane sugar and in the fully imbibed intact seed, Crocker and Davis (4) estimated that the embryo in the imbibed seed exerted a pressure of nearly a hundred atmospheres on the coats. This is unsatisfactory in that it is an estimate rather than a direct measurement; also they did not figure on lateral swelling which might compensate in part for longitudinal swelling. At least they showed that the swelling embryo pressed against the coat with considerable force but with insufficient force to break the coat.

Barton (1) reviewed the literature on the germination of nuts of various Juglandaceae and reported the following conclusions from her experiments in which she worked entirely with intact nuts. Pretreatment of black walnuts and butternuts for two to four months in a moist medium at a low temperature was necessary for seedling production. When a sufficiently long period at about 3° C. was preceded by one to four months at a high temperature (21° C.) germination was hastened but not increased. Seed-

ling production by hickory nuts was improved by pretreatment in moist soil for one to five months at 3° or 10° C. but some seedlings were produced without pretreatment. Fall planting under a mulch outdoors gave good seedling production in the spring in all three forms. Muenscher and Brown (15) found that nuts of *Juglans* species husked soon after harvest, before they were completely air-dried, and stored in moist peat at 1° to 3° C. for five to six months had their dormancy broken and remained viable for at least three months thereafter. The method was effective on all ten species tested and avoided rodent destruction which is experienced in fall planting outside. Heit (13) gives the best time for fall planting of various nuts and other tree seeds, also states whether removal of husks and mulching are desirable for the several species. Six to seven months' stratification at 4° C. gives best results (12) with black walnut and butternut.

Hagerup (11) says that in the *Corylus* (*C. avellana* and *C. maxima*) embryo four elongations (auricles) project from the base of the lamina of the cotyledons which entirely surround the root like a sheath. At germination these split the hard fruit, growing rapidly in length and thickness and also bending outward. The stalk of the cotyledons grows considerably in length and thus pushes the embryonal bud out of the shell. Rowlee and Hastings (16) describe the embryos and seedlings of walnut, hickory nut, and butternut, and several other Amentiferae. In the nuts each of the valves of the shell contains one-half of each of the cotyledons so that when the shell is split in germination the split is at right angles to the greatest expanse of the cotyledons. In germination the shell splits from the micropyle and the tip of the radicle and two or four basal lobes of the cotyledons push out together. The stalks of the cotyledons lengthen and carry the plumule out of the shell.

#### MATERIAL AND METHODS

Seeds of black walnut (*Juglans nigra* L.), hickory nut (*Carya ovata* [Mill.] K. Koch), and butternut (*Juglans cinerea* L.) were used in these experiments. The walnuts were collected both from individual trees and from groves of 6 to 8 trees near West Grove, Pennsylvania, and the hickory nuts were collected from individual trees near West Grove and Canton, Pennsylvania. The butternuts were obtained from Herbst Bros., New York, N. Y., as part of their 1943 crop and tested only during the winter of 1943-1944. The walnuts and hickory nuts were first collected December 5, 1943 and the experiment started December 13 and continued until the following May, 1944. Because of the nature of some of the results, it was then decided to repeat the work the following year with nuts harvested October 26, 1944, to insure that the cold temperatures to which the 1943 crop had been exposed before harvest had not unduly influenced the results obtained.

Walnuts were freed of the green hulls by passing through a corn sheller

so adjusted that the metal spurs would not injure the surface of the nut. The hickory nuts were shellbark and therefore free of the hulls when harvested. Butternuts had to be soaked to free the nut from the dried hull in order that it could be removed by hand rubbing. This preliminary cleaning was accomplished immediately after harvesting, then the nuts were shipped to Yonkers, N. Y., by express. Upon arrival the nuts were placed in water and most of the remaining hull was rubbed off by hand. All nuts floating at the time of cleaning were discarded since these nuts were incompletely developed and unfit for experimental purposes. This floating test was also used to separate the undesirable hickory nuts. This method is not reliable for separation of nuts if they have dried to any extent after harvest. Various degrees of cleaning of walnuts were tested. This will be discussed with the results of the experiment.

It was important that the nuts did not dry too much before they were placed in the experiment since the shells cracked and were rendered useless for experimental purposes. This was especially true of hickory nuts which may dry sufficiently on the ground after falling from the tree, and before gathering, to cause cracks to form in the shells either at the dehiscent layer or at random over the exposed surface. This difficulty was not encountered with walnuts until after the nuts were thoroughly cleaned and held in the laboratory for a week or more. Since the nuts used in these experiments were cleaned and immediately placed under treatment, no drying cracks were experienced in the tests. The nuts were graded into fairly uniform sizes by discarding the very small ones, and none was used that showed any visible injury to the shell.

The cleaned seeds were buried in a good compost garden soil in flats and placed in rooms held at the several temperatures mentioned below. Care was taken in spacing the nuts so that they were not in contact with each other and the soil was watered to maintain it in good condition for plant growth. The temperature of the soil rather than that of the room was recorded.

Besides the intact nuts used for pressure tests, other nuts were partially opened by means of a vise, hack saw, and small chisel, in order to bare the radicle and surrounding cotyledonary tissue without causing injury to the living tissue. This was difficult to accomplish without causing injury to a high percentage of the embryos. However, the slightly injured embryos that were not readily observed to be injured before placing in soil for storage, soon molded and could be discarded early in the experiment. If the living tissue had not been injured when the shell was sawed and broken away, the exposed embryo did not mold during storage in the soil. A photograph of the exposed radicle end of the embryo and the wedge of shell removed from the radicle region is shown in Figure 1 B.

Germination tests were made in moist peat in small containers placed

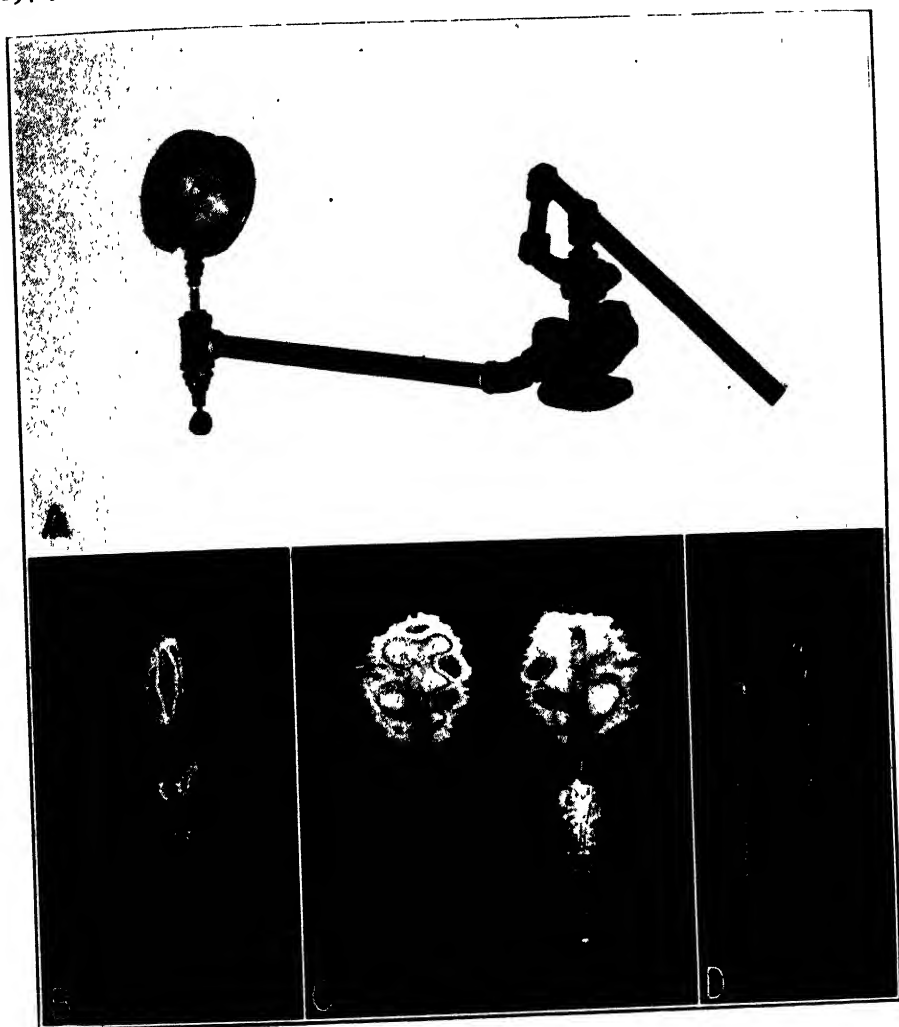


FIGURE 1. The methods of handling the nuts for various determinations including the apparatus used are shown. A. Hydraulic pump to develop high pressure, 1000-pound gauge for recording pressure, and attached nut are shown in assembly. Water used to develop the pressure was drawn from a reservoir that is not shown. B. Wedge-shaped portion cut from shell over the embryo. C. Section of walnut showing hole bored through shell and into embryo tissue without cutting a second portion of shell. Also is shown special threaded pipe screwed into shell to permit the development of pressure necessary to break shell. D. High speed steel drill and threading tap used to bore and thread the shells.

in electrically controlled ovens at temperatures of 1°, 5°, 10°, 15°, 20°, 25°, 30°, 35° C., and in flats of garden soil placed in large controlled temperature rooms at soil temperatures of 6°, 11°, 17°, 23°, and 28° C. As soon as seeds germinated the seedlings were planted in 4-inch pots and placed in the greenhouse for later growth measurements.

The breaking pressure of the nuts was determined by first boring a hole, with a high speed electric drill, perpendicular to the dehiscent layer of the nut and at such an angle that the drill would not cut any portion of the shell other than that through which it entered. A No. 16 (0.177 in.) drill was first tried but for most of the experiments a smaller drill, No. 30 (0.1285 in.), was used. Following the boring of the hole, threads were worked in the shell by a tap (A.S.M.E.) size 12-24 for the larger and 8-32 for the smaller hole (Fig. 1 D). The nut was then threaded on a tapered attachment to a hydraulic pump as shown in Figure 1 C. The reason for the tapered thread was to insure a tight fit between the metal and the shell that would not leak water and a fit that would withstand the pressure to be exerted in the nut. By experience one was able to determine the correct tightness of threading the nut on the tapered thread without cracking or injuring the shell so it would not break at that point, or be forced off the threads by the pressure applied.

The pressure was applied to force the shell open by pumping water into the nut. The apparatus shown in Figure 1 A consists of a hydraulic pump with appropriate valves, piping, (a water reservoir not shown) and an accurately calibrated gauge reading to 1000 pounds. With the entire system full of water and the head holding the nut attached, pressure was applied by working the plunger to force more water into the system until there was obtained a sharp and distinct breaking of the shell. The maximum reading of the gauge just previous to the break was taken as the breaking pressure.

In the first set of experiments, breaking pressures were determined on 10 nuts for each storage period and condition. Samples were taken after 0, 7.5, and 15 days of planting and on 15-day intervals thereafter up to 120 days. For the second year 25 nuts were used for each breaking sample and the tests were made at 15-day intervals from 0 to 150 days. Special tests were set up to investigate various phases of this problem. These will be discussed with the experimental results.

Because of the large number of individual pressure determinations made, it was impossible to record the data in tables. Consequently, averages and graphic means are used to show the results of these investigations. In preparing the curves the "moving average" method of Camp (2, p. 103) was used, 3 being taken as the value of "K." The average breaking pressure for each of three contiguous storage periods was obtained and was

plotted as an ordinate over the average of the storage periods in days that gave these values.

Because of the method of calculation used the graphs begin at 15 days and end at 135 days although the total duration of the experiments was 150 days.

## EXPERIMENTAL RESULTS

### PRELIMINARY EXPERIMENTS

The early experiments show that black walnuts broke with lower pressure when dry than when wet. Nuts that had been stored in a cool basement for one year were soaked in water for 48 hours during which time they took up 12.6 per cent water and broke with an average pressure of 400 pounds as compared to the air-dry nuts that broke with an average pressure of 173 pounds. This is opposite to the findings of Müller (14) for *Corylus* and *Pinus* shells. Hickory nuts were examined to test this difference, but it was found that all dried nuts had cracked at the dehiscent layer. Peach pits subjected to internal water pressure emitted a stream of water through the micropyle and could not be used in these studies. When walnuts were subjected to air pressure while under water, it was found that minute bubbles appeared all over the shell as the pressure approached 100 pounds, which indicated that the shell has many fine perforations. These may further the absorption of water by the embryo. They also made questionable the use of air pressure in these experiments. Under sufficient air pressure the walnut shell broke into small fragments instead of merely at the dehiscent layer. The fragments were thrown through the air with great force, requiring a guard to protect the worker. Water absorption was not a limiting factor in the germination nor in the ultimate decrease in the breaking pressure of the dehiscent layer of the black walnut. This was also borne out by removing the shell over the radicle end of the embryo, then placing both cut and uncut walnuts in moist soil, followed by moisture determination of the embryo tissue during various periods of storage up to 42 days. The results of these tests showed that there was no difference in the rate of moisture absorption by the embryo whether the shell was intact or opened. Walnuts appeared to be in equilibrium with moist soil when the embryo had absorbed 30 per cent moisture.

### BLACK WALNUT

*Breaking pressure experiments.* The average breaking pressure of the walnuts at the start of the experiment was 627 pounds. With continuous storage in moist soil at 11°, 17°, 23°, and 28° C. the breaking pressure decreased but it was not greatly altered with storage at 6° C. Since the results were found to be similar for both years of study only the data from

the 1944 crop, the larger sample lots, were used to make up the curves in Figure 2, drawn by the method of "moving averages." Although the breaking pressure dropped most rapidly with moist storage at 28° C., the intact walnuts did not germinate at this temperature. At the lowest temperature of 6° C., where the breaking pressure changed least, the walnuts started to germinate by the end of the storage period of 150 days. The 1944 crop of walnuts was slower to germinate in low temperature storage, 6° and

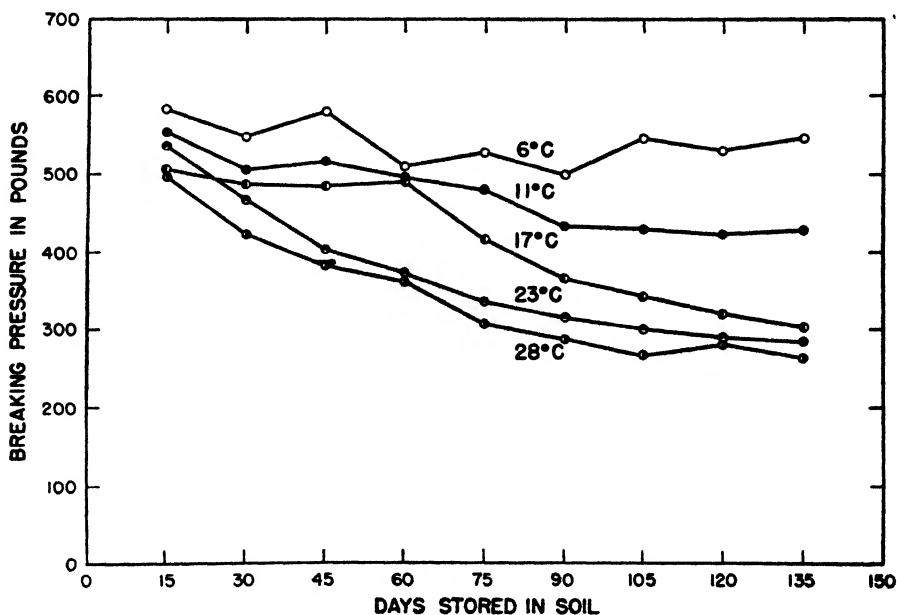


FIGURE 2. Changes in breaking pressure (moving averages) of walnuts as a result of storage in moist soil at various temperatures.

11° C., than the 1943 crop. This is thought to be due to the fact that the nuts in the 1944 crop were green when the experiment started. The nuts in the 1943 crop started to germinate after 90 days at 6° and 11° C. Probably these nuts had matured more completely before being placed in moist soil.

While studying the breaking pressure data, it was discovered that the standard deviation from the mean and of course the coefficient of variation (which averaged 38.5 for all lots) of any lot of 25 pressure determinations were not reliable because each lot appeared to be a mixed population with two separate frequency distributions. In Figure 3 are shown five histograms produced from all the breaking pressure data for all sampling periods at each temperature. The histogram for the 6° C. storage shows that the walnuts appeared to consist of a mixture of two groups, one group breaking

at a high pressure with a mean of 824 and a second group breaking at a lower pressure with a mean of 408. The mean for the entire lot of walnuts was 550 pounds pressure which falls approximately midway between the two peaks of the histogram. The data showed that the frequency with which the pressure occurred in the higher group changed very little as the storage period progressed over to 150 days so that at the end of the experiment there were a few nuts still breaking at a pressure of from 750 to 1000 pounds. With increase of storage temperature to 11° C. slightly fewer nuts were found breaking at the higher pressure as indicated in the histogram (Fig. 3), and the mean had dropped to 746 pounds for the high group and 365 for the low pressure group. Also it was found that the frequency of breaking in the higher group diminished from the 120-day period.

With continued increase in temperature of storage, 17°, 23°, and 28° C., there was but little change in the means of the high pressure groups and a steady decline in the means of the low pressure groups. Although care was exercised in calculating these means they are only approximate, since the two populations no doubt overlap, it would be difficult to determine just what to include in each of the two groups of pressures for each temperature. However, as seen in Figure 3, the histograms indicate a skew curve due to the fact that there was an unequal distribution of the breaking pressures as a result of mixed population of nuts. Significant is the fact that the breaking pressure data showed the proportion of nuts which were distributed into the high pressure groups becoming smaller with 75, 45, and 15 to 30 days of storage at 17°, 23°, and 28° C., respectively. With the knowledge that the walnut is conditioned for germination by moist storage at 6° and 11° C., one would expect to have found the pressure required to break the shell at these temperatures to have fallen more rapidly than at the higher temperatures where germination is not usually found to occur.

Since no differences were found in breaking pressure of walnuts thoroughly cleaned, and roughly cleaned by mechanical brushes, and those being rubbed free of hulls by hand, tests were made with nuts firmly enclosed in the green hull. The unhulled nuts were placed in soil at 6°, 17°, and 28° C., and samples were removed after 45 and 90 days. The breaking pressure data show that at 6° C. the nuts broke at higher pressures with extended storage during which time there was only a little softening of the hulls. At the end of 90 days, 30 per cent of the nuts broke at a pressure in excess of 1000 pounds and the average breaking pressure of the lower group was 510 pounds, compared with a breaking pressure of 588 pounds at the start of the test. At 17° and 28° C. the hulls loosened rapidly and quickly disintegrated and the breaking pressure fell as already shown in the graph (Fig. 2). These results suggest that the intact hulls prevented the loss of a sealing substance from the dehiscent layer.



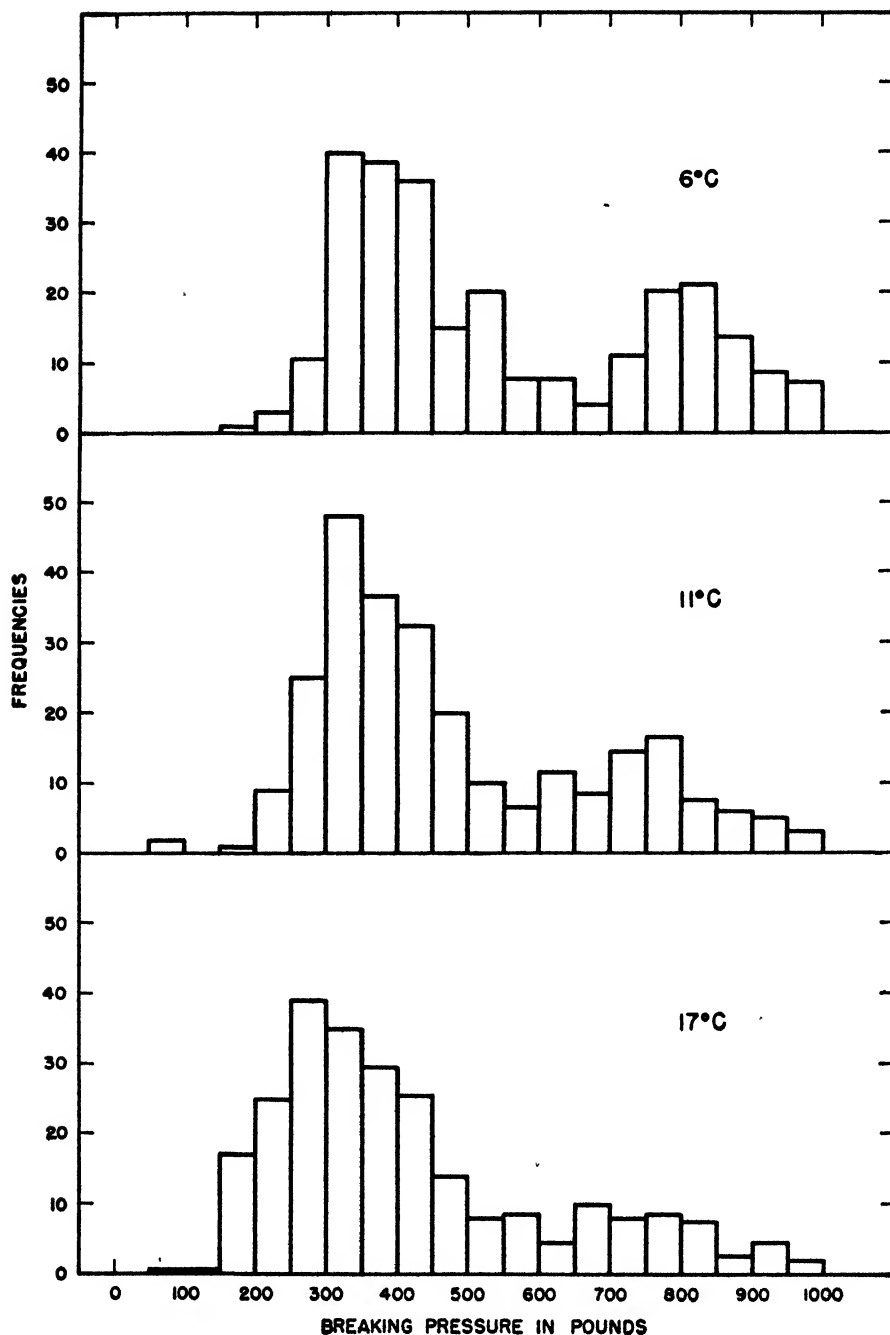


FIGURE 3. See legend on opposite page.

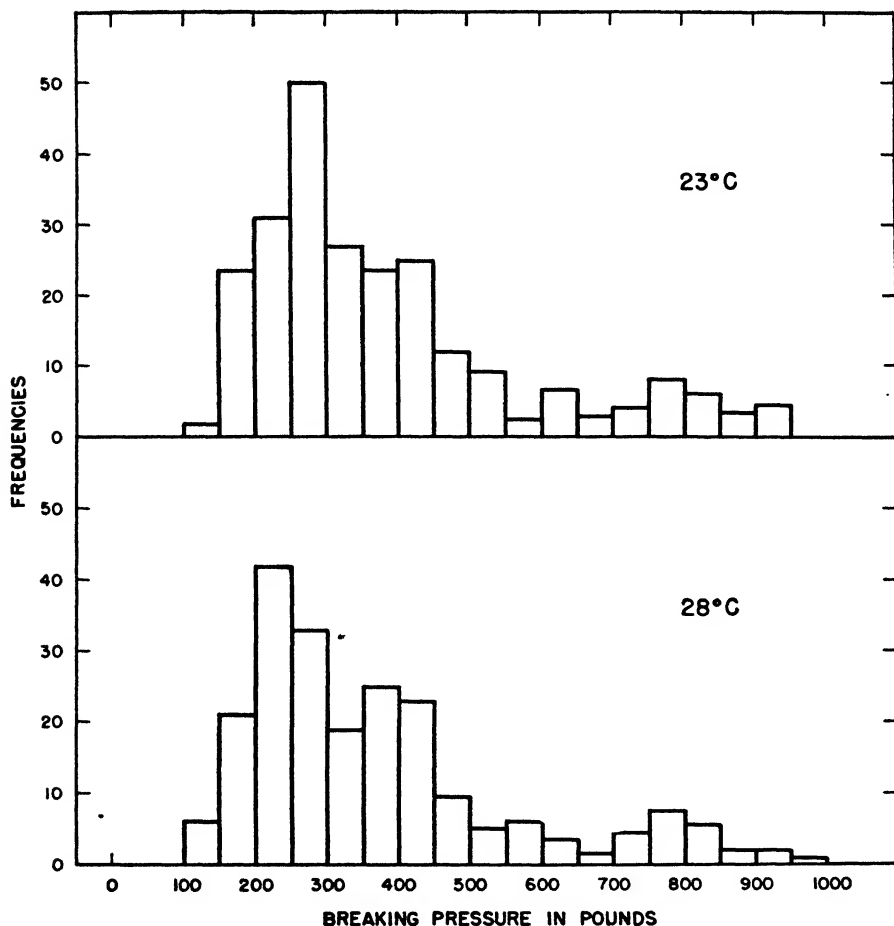


FIGURE 3. Frequencies with which walnuts were broken by various pressures applied following storage at various temperatures. These histograms were prepared by taking all the breaking pressure data obtained from each storage temperature, 25 nuts used per sample and 11 tests made during the period of the experiment.

Machine-cleaned walnuts were next placed in Mason jars filled with distilled water, in jars containing 1-1000 mercuric chloride in distilled water, and in distilled water without rinsing after the shells had been standing in calcium hypochlorite (15 g. in 130 cc.  $H_2O$ ) for 15 minutes. The jars were sealed after temperature adjustment to storage room and held at 5° and 20° C. Sufficient numbers of lots were set up to permit removing of sealed samples at the end of 7, 15, 30, 60, and 90 days of storage. The results of all of the pressure tests show that there was no significant change in the breaking pressure of the nuts from the start when held under any of the above conditions of storage. This was also true for the walnuts

held in air-dry storage (85 per cent rel. humidity) at 5° C. Walnuts held in air-dry storage (approx. 20 per cent rel. humidity) at 20° C. were cracked within one week and could not be used in these tests. Thus it was evident that the adhesive material holding the two halves of the walnut together was not altered by storage in water. Likewise this substance was not destroyed when the shells were sterilized or held in solution that prevented the growth of organisms. Freedom of these walnuts from organisms was proven when opened by placing the halves on potato dextrose agar and incubating at both 20° and 35° C. for 10 days.

Further tests of the effect of moist storage at 17° C. for 30 and 60 days followed by low temperature storage at 6° C. had no noticeable effect upon the breaking pressure of walnuts tested every 15 days up to 120 days. In general the averages of the pressure data followed the general trend shown by the groups in Figure 2. During storage at 17° C. the breaking pressure dropped, of course, to a greater extent during the 60-day storage than for the 30-day period, the average breaking pressures being 323 and 450 respectively. With storage at 6° C. the breaking pressure of the 30-day pre-stored nuts continued to drop gradually with storage since the values were still high when the nuts were removed to 6° C. However, the nuts held for 60 days at 17° C. were at a relatively lower pressure when removed to 6° C. and did not change thereafter. A further interesting observation was that the walnuts in moist soil for 30 days at 17° C. before being placed in moist soil at 6° C. required 90 days at the latter temperature before they started to germinate and the breaking pressure of this lot was 337 pounds. Walnuts stored 60 days at 17° C., then removed to 6° C., began germination in 15 days. The breaking pressure of this lot of nuts was 385 pounds. Although high temperature storage had reduced the breaking pressure of the shells, the results of these tests indicate that the conditioning of the embryo for growth in the intact shell was of more importance than the reduction of the breaking pressure of the shell.

This idea is further brought out by the data in Table I which shows that if the embryo has been conditioned for growth for 105 days at 6° or 11° C. it will begin to grow even when the breaking pressure of the nuts remains relatively high. If the embryo is not conditioned for growth because of high temperature storage at 17°, 23°, or 28° C., it will not grow even though the breaking pressure of the nuts has fallen to a relatively low value. Just how the embryo can develop the pressure necessary to force open the shell at the dehiscent layer has not yet been determined.

*Chemical analyses.* Chemical analyses of the embryos of walnuts stored in moist soil at 6°, 11°, 17°, and 28° C. were made with the result that no significant differences were observed in the reducing sugar or sucrose content of the embryos, when sampled at 15, 30, 60, and 90-day intervals. The walnuts used in these experiments had been stored at 10° C. in a

TABLE I  
WALNUTS STORED IN MOIST SOIL AT VARIOUS TEMPERATURES, THEN REMOVED TO  
GREENHOUSE FOR BREAKING PRESSURE TESTS AT VARIOUS INTERVALS WHILE  
WAITING FOR GERMINATION

105 Days in moist soil at ° C.	Breaking pressure in pounds and number germinating after removal to greenhouse at 24° C. for days				
	0	3	6	12	24
6	320	320	280	320	
	680	740			
	520	280			
	700				
	3G*	3G	5G	5G	6G
11	400	280	280	160	240
	560	480	200	260	320
	320	320	200	300	320
	500		500		
	320				
17	280	3G	2G	3G	3G
	400	280	220	290	200
	280	220	220	360	280
	320	240	360	230	340
	440	220	360	280	190
23	320	240	200	210	260
	320	220	—	230	—
	150	320	220	160	240
	220	200	360	190	240
	150	190	200	240	190
28	160	200	280	190	200
	240	340	—	190	240
	240	—	—	170	—
	190				
	180	180	180	120	240
28	200	240	340	520	280
	230	240	160	160	280
	360	160	360	190	280
	260	—	160	210	—
	200	—	—	240	—

— The nut slipped and the drill cut too deeply, or an enlarged hole that could not be properly threaded.

\* G preceded by number = nuts germinating in that sample.

relatively air-dry condition (85 per cent relative humidity) for two months previous to being placed in this experiment. The moisture content of the embryo and storage tissue was 7 per cent at the start and increased to 20 per cent in 15 days and to 30 per cent at the 30- and 90-day periods of storage in moist soil. The fat content of the walnuts dropped from 60 per cent at the start to approximately 40 per cent (based on wet weight) at the end of 90 days, the most rapid change being observed in the lots stored for 15 days at 28° C. where a 12 per cent reduction in fat took place. This reduction was just three times that observed at the other three tempera-

tures. Further reduction in the fat content took place at a gradual rate at all temperatures, except 6° C. where at the 30-day interval it had reached its lowest value for the period of study and all lots sampled from this temperature after this interval gave approximately the same fat content. Analyses of the fat (i.e., refractive index, iodine No., saponification No., and soluble and insoluble acids) showed no significant changes with longer periods in moist soil. These results do not offer a solution to the pressure developed to break the walnut shell.

*Germination tests.* Since there appeared to be no direct relationship

TABLE II  
PERCENTAGE GERMINATION AND DAYS REQUIRED FOR TWENTY-FIVE WALNUTS HARVESTED  
IN 1943 TO GERMINATE WHEN THE SHELL WAS EITHER INTACT OR CUT AWAY  
FROM THE EMBRYO TISSUE

Soil temperature at °C.	1943 Harvest planted in garden soil			
	Open		Intact	
	% Germ.	Days	% Germ.	Days
6	50	74	29	148
	100	91	29	160
11	50	41	50	105
	100	57	67	113
			67	160
17	50	31	0	160
	100	135		
23	50	113	0	160
	72	135		
	72	148		
28	50	41	0	160
	64	57		
	100	170		

between germination of walnuts and breaking pressure of the shell or chemical analyses of the entire embryos, the germination behavior of embryos was next studied when the restricting action of the shell was removed over the radicle end of the embryo in contrast to the germination behavior of intact nuts. Removing the portion of the shell over the radicle (Fig. 1 B) allowed germination of the walnut at temperatures ranging from 6° to 28° C. in comparison with no germination of intact walnuts at 17° C. or above and a slower rate of germination at lower temperatures as is shown by the data in Table II. Opened walnuts held in soil at 11° C. gave 50 per cent germination in 41 days and 100 per cent by the 57-day period in comparison to 105 days for 50 per cent germination of intact walnuts and a maximum germination of 67 per cent in 160 days. At 28° C. the opened

walnuts produced 50 per cent germination in 41 days as compared with no germination of the intact walnuts in 160 days. The opened lots stored at 23° C. were erratic for some unknown reason in both the time required for germination and the total percentage of germination obtained during the test. Because of the wide range between the temperatures of storage used for the soil tests, other tests with closer temperature treatments were studied using a limited number of nuts in moist peat.

TABLE III

PERCENTAGE GERMINATION BASED ON NUMBER OF NUTS WHICH DID NOT ROT AND DAYS REQUIRED FOR WALNUTS HARVESTED IN 1943 AND 1944 TO GERMINATE WHEN THE SHELL WAS EITHER INTACT OR CUT AWAY FROM THE EMBRYO TISSUE

Temp. °C.	1944 Harvest: 10 nuts in peat Nov. 9				1943 Harvest: 7 nuts in peat Jan. 12			
	Open		Intact		Open		Intact	
	% Germ.	Days	% Germ.	Days	% Germ.	Days	% Germ.	Days
1	0	211	0	211	—	—	—	—
5	0	211	0	211	57 100	59 84	—	—
10	50 100	69 84	20 20	133 211	57 100	42 55	—	—
15	50 100	106 117	50 90	133 204	50 100	15 42	—	—
20	30 30	69 211	0	211	43 71 71	15 20 147	0	147
25	30 30	13 211	0	211	50 60 60	10 15 147	14 14	28 147
30	67 67	8 211	0	211	20 20	10 147	0	147
35	29 29	13 211	0	211	0	147	0	147

Table III shows the comparable germination of black walnuts of the 1943 and 1944 crops at eight different temperatures with the coats intact and open. The two lowest temperatures, 1° and 5° C., were apparently too low to give germinations in the 1944 crops with either open or intact nuts, but 5° C. gave good germination in the 1943 crop, opened. Both 10° and 15° C. gave complete to fair germination in 1944 crop, both intact and open, and complete germination in the 1943 crop, open. No tests were

made for the 1943 intact at 1°, 5°, 10°, or 15° C. The four temperatures, 20°, 25°, 30°, and 35° C., gave some germinations in the 1944 crop, open, and all but 35° C. likewise for the 1943 crop, open. No germinations occurred in either crop intact at temperatures 20° C. or above except for one nut at 25° C. This table emphasizes again the fact that open nuts germinate over a wide range of temperatures, while intact nuts germinate only at lower temperatures, no difference how long they are in moist soil and this in spite of the fact that the shells are greatly weakened by long periods in moist soil at high temperatures.

After three months in moist peat at 20°, 25°, and 30° C. some of the intact nuts were opened and placed back in moist peat at the respective temperatures with the result that all uninjured nuts germinated readily and produced good seedling growth in the greenhouse. This shows that long periods in the germinator at high temperatures with intact shells preventing germination do not throw the embryos into dormancy. It also seems to add some evidence that mechanical strength of shells may prevent embryos that are ready to germinate from growing but before this explanation is accepted fully it must first be determined whether inhibiting chemicals accumulate in intact nuts in the moist soil at high temperatures. At low temperatures this certainly does not occur to a degree that prevents the embryos from growing, splitting the shells, and germinating.

Upon finding that germination of opened nuts took place at somewhat reduced rate at the higher temperature, it was considered necessary to determine if such treatment would cause the development of dormancy in the walnuts. Tests were set up with large quantities of opened walnuts in order that all quickly germinating nuts could be discarded leaving sufficient uninjured and slow-to-germinate nuts to conduct the test, the data of which are shown in Table IV. The results of these tests show that long continued storage in moist soil at 20° C. does not retard the germination of opened nuts when placed in a greenhouse at 24° C. If after 90 days' storage at 20° C. the opened nuts were held for 30 or 60 additional days at 6° C. previous to placing in the greenhouse the germination was retarded. Storage of the intact nuts for long periods at 20° C. allowed for the reduction in breaking pressure so that with additional storage at 6° C. the intact nuts began to germinate at earlier dates than when stored continuously at 6° C. Here again we are working with mixed samples of walnuts besides the fact that many unopened nuts start to rot during the long period of storage at high temperature.

*Growth of walnuts in greenhouse.* Ten walnuts were planted individually in pots of garden soil from each test shown in Table IV and growth measurements were made on the plants developed at various intervals for a period of six months. The results show that regardless of the temperature of the germinators all uninjured opened walnuts produced seedlings that

grew to all appearance normally without extreme dwarfishness such as is found in several rosaceous plants grown from embryos that have not had low temperature after-ripening (8, 10) or from cocklebur embryos (17) in secondary dormancy without such after-ripening. There was great variation in the vigor of growth of seedlings from any one treatment but as

TABLE IV

DAYS REQUIRED FOR GERMINATION OF OPEN AND INTACT WALNUTS IN GREENHOUSE AT 24° C. AFTER THEY WERE FIRST KEPT IN MOIST SOIL AT 20° C. FOR DIFFERENT PERIODS, FOLLOWED BY VARIOUS PERIODS IN MOIST SOIL AT 6° C. TEN WALNUTS WERE USED IN EACH REMOVAL PERIOD

Days in soil at 20° C.	Condition of coat	Days at 6° C.											
		0		15		30		60		90		100	
		% Germ.	Days	% Germ.	Days	% Germ.	Days	% Germ.	Days	% Germ.	Days	% Germ.	Days
0	Open	100	85	100	41	100	27	100	10	100	23	—	—
	Intact	0	210	10	210	50	178	50	38	80	37	40	40
45	Open	100	54	*	*	100	21	100	21	100	25	—	—
	Intact	0	210	20	99	30	38	40	55	70	25	90	28
90	Open	100	70	*	*	100	42	100	60	—	—	—	—
	Intact	10	37	10	56	30	42	40	60	80	28	—	—

Days at 20° C.													
Continuous**	Open	100	85	†	†	†	†	100	98	100	70	—	—
	Intact	0	210	0	210	10	99	20	66	10	37	20	90

— Insufficient nuts to finish tests.

\* To conserve samples for later tests no samples were made here.

\*\* No storage at 6° C. but removed to greenhouse at each 6° C. storage interval.

† Already germinating at 20° C., samples of 10 germinated nuts were transferred to greenhouse for growth studies.

between treatments there was no measurable difference except for opened nuts held in low temperature germinators for long periods. These seedlings averaged about 2 inches greater growth in height than those held in high temperature germinators. With this exception, all seedlings were similar as to color, shape, and size of leaves and plants.

Seedlings grown from intact nuts, if germination was possible at any temperature or combination of temperatures, were always taller by 2 to 6 inches than seedlings produced from opened nuts. Marked differences were observed with walnuts stored for various periods at 6° C. following



90 days' storage in soil at 20° C. in that progressive increase in height of plants was found with extended periods of storage at the lower temperature.

From these results it is apparent first, that the walnut embryo is not dormant but will grow and produce a plant if the shell is removed over the radicle; second, that something is lost from the embryo when the nut is opened and stored at 20° C. which retards slightly the growth of the plant

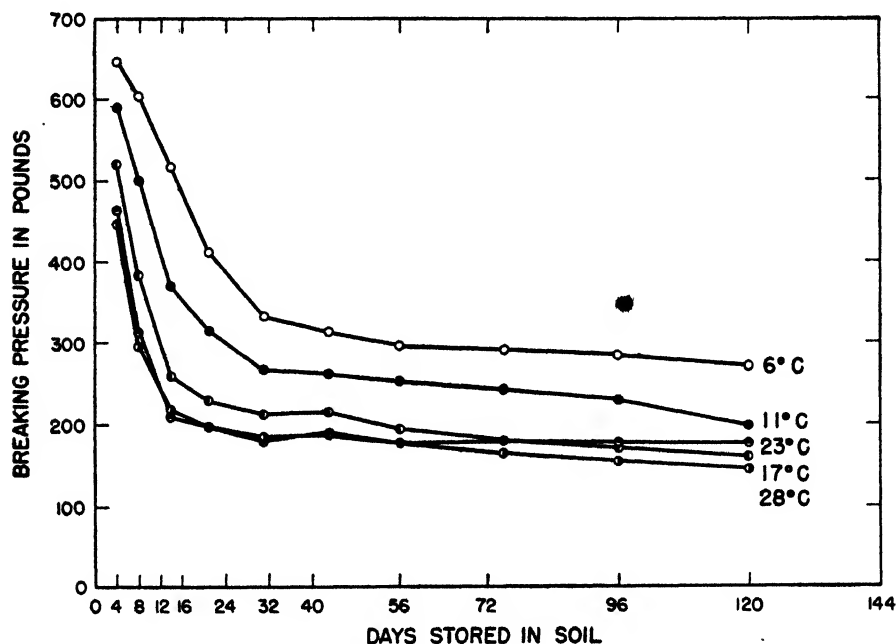


FIGURE 4. Changes in breaking pressure (moving averages) of hickory nuts as a result of storage in moist soil at various temperatures.

when compared with the unopened nut; and third, the walnut embryo is improved in its germinating capacity and seedling development if the embryo is first "conditioned for germination" by a period in a low temperature germinator irrespective of whether the shell is opened or intact. In all cases the increased growth brought about by low temperature conditioning was slight, especially so in comparison with that shown by certain dormant rosaceous and secondarily dormant *Xanthium* embryos.

#### HICKORY NUT

*Breaking pressure experiments.* The average breaking pressure of hickory nuts when they were received from Canton and West Grove, Pa., areas in 1944 was 670 and 660 pounds respectively. With storage in moist

soil the breaking pressure decreased quite rapidly during the first 16 days. However, it was found here as with walnuts that the breaking pressure of nuts in a germinator at 17°, 23°, or 28° C. decreased more rapidly than those held at 6° or 11° C. A typical experiment with hickory nuts gathered from one tree at Canton is depicted in the "moving averages" curves shown in Figure 4. Because of the slowness of germination (due no doubt to very early harvesting of these nuts) the authors were able to study the

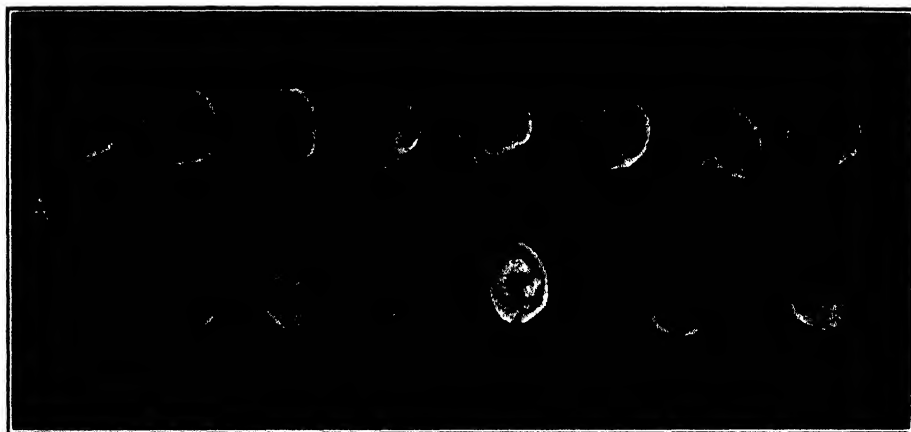


FIGURE 5. Early-harvested hickory nuts subjected to high pressures broke (A) at the side rather than at the dehiscent layer but after 4 days' storage in moist soil they broke (B) at the dehiscent layer.

breaking pressures over a period of 114 days which allowed for a spread in the temperature effects. As the temperature of moist storage dropped below 23° C. there was a general slowing up of the reduction in breaking pressure with duration of storage.

As already discussed with walnuts, it was found also with hickory nuts that breaking pressure data represented the results from a mixed population having in itself two separate means. Histograms prepared from the data appeared in general similar to those shown for the walnuts in Figure 3. In the case of the hickory nuts the mean of the upper pressures at all the temperatures of storage was in the neighborhood of 650 pounds and of the lower group 200 pounds. Differing from the walnuts, however, the high pressure group of the hickory nuts rapidly shifted over into the low pressure group. The time and pressure relationships are shown in approximation in Figure 4. The actual values from which these curves were made show that the time required to reduce the breaking pressure to 300 pounds was 7, 7, 10, 18, and 42 days at 28°, 23°, 17°, 11°, and 6° C., respectively.

Significant is the fact that besides a record of the actual pressure at

which the hickory nut broke there was observed a rapid change in the place at which the nuts broke as the result of the pressure applied. When the Canton hickory nuts were first tested a large proportion broke from the side as shown in Figure 5 A rather than at the dehiscent layer separating the two halves of the shell as shown in Figure 5 B. This result indicates that for these early-harvested nuts the material sealing the two halves together is stronger than the rest of the shell and that if the shell had not given way the breaking pressure at the dehiscent layer would have been higher than was recorded. After 4 to 8 days of moist storage at any temperature the nuts always broke at the dehiscent layer. This situation did not occur with West Grove hickory nuts, probably due to the greater maturity and dryness at the time of harvest and before testing for breaking pressure.

As already mentioned, the original breaking pressure of the nuts harvested at West Grove was about the same as those harvested at Canton in spite of the difference in the region of the break. These nuts differed from those harvested at Canton in that germination took place more rapidly so that the experiment had to be discontinued at the end of 64 days. As shown in Figure 6, the breaking pressures at all temperatures of storage dropped rapidly. During the period in moist soil at 28° C., 56 per cent of the nuts germinated in 8 days and 88 per cent in 16 days. At 17° C., 40 per cent were germinating after 32 days and 80 per cent after 64 days. The nuts stored at 6° C. started germinating in about 80 days. The interesting fact is that the unsmoothed curves in Figure 6 are in general the same as those shown in Figure 4 and that there is no sharp break in the curves to indicate any great change in the breaking pressure of the nuts when germination took place.

Hickory nuts harvested at West Grove late in the fall of 1943 and from the same tree as those used in the experiment shown in Figure 6 gave in general the same picture in regard to the breaking pressure changes with the moist soil treatment. Although the breaking pressure at the start was only 300 pounds, due to lateness of harvest and the fact that the nuts were exposed to conditions favorable for reducing breaking pressure, there was still a marked lowering of pressure before germination started. At 28° C. the average pressure was reduced to 230 pounds in 8 days and germination was observed with many of the nuts in 15 days when the average breaking pressure of the remaining nuts was 200 pounds. In storage at 17° C. the average pressure decreased to 275 pounds in 8 days and to 155 pounds in 45 days when 50 per cent of the nuts were germinating. At the 30-day period these nuts showed a breaking pressure of 230 pounds. At 6° C. the average breaking pressure was 213 pounds after 105 days in moist soil when 70 per cent of the nuts were germinating. The results at temperatures of 23° and 11° C. were in between the results of 28° and 17° C.

and 17° and 6° C. respectively. Apparently the reduction in breaking pressure takes place irrespective of how the nuts are handled provided the moisture conditions are favorable.

In another experiment where the hickory nuts were held in water both with and without sterilization of the shell there was found no significant change in the breaking pressure during the 150 days of storage at either 5° or 20° C. This test was conducted in the same manner as the walnut test mentioned earlier and with the same apparent results that the pres-

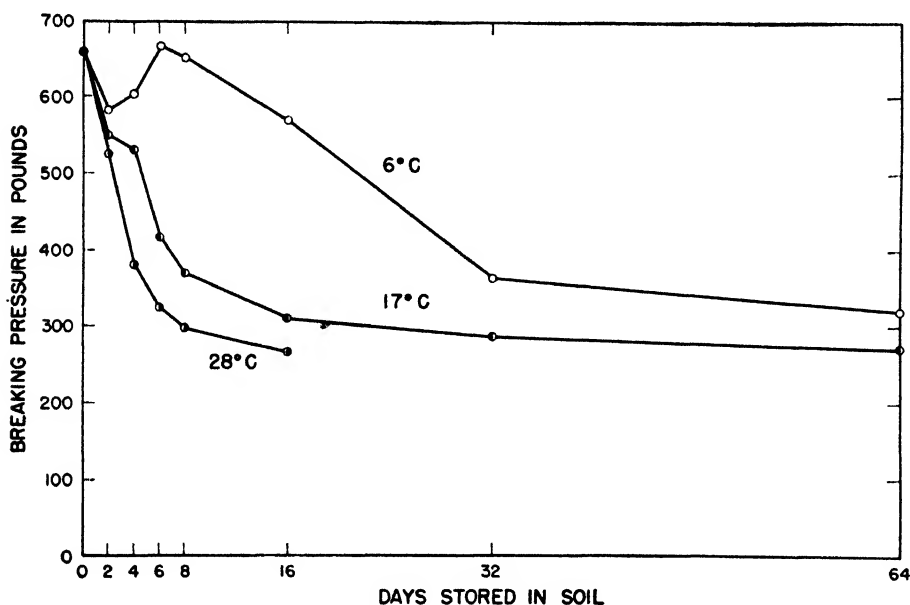


FIGURE 6. Breaking pressures of hickory nuts stored in moist soil at various temperatures.

ence of the water alone does not weaken the sealing substance in the desiccant layer.

For the 6° C. moist storage it will be noted (Fig. 6) that the breaking pressure falls during the first two days and then rises again to a maximum on the 6th day after which there is a gradual fall. This fall and later rise in breaking pressure at 6° C. in moist storage held for all six lots of hickory nuts run during the course of the experiments. This behavior was also observed in a lot of 2-year-old hickory nuts originally collected at Canton, Pa., that had been stored under conditions in which the shells had not cracked but the embryos had lost their vitality. When these nuts were soaked for 12 hours, they had a breaking pressure of 660 pounds which decreased in 3 days to 350 pounds, then rose to 380 pounds by the 5th day, and it was still 380 pounds on the 12th day, following which it decreased

gradually to 150 pounds by the 48th day. At the 48th day of storage the embryo tissue was badly decomposed. Nuts stored at 28° C. gave a straight line drop in breaking pressure to 100 pounds on the 12th day when the embryo tissue was found to be decomposed. Just what conclusions may be drawn from these observations are at present uncertain. However, the

TABLE V  
GERMINATION OF HICKORY NUTS FROM VARIOUS TREES WHEN HELD IN MOIST PEAT AT CONTROLLED TEMPERATURES

Temp. °C.	Days required for percentage germination* of hickory nuts harvested at											
	Canton, Pa.								West Grove, Pa.			
	Tree 1**		Tree 2**		Tree 3**		Tree 4**		Tree 5†		Tree 6††	
	%	Days	%	Days	%	Days	%	Days	%	Days	%	Days
1	0	262	0	262	0	262	0	262	0	242	0	220
5	0	262	0	262	0	262	0	262	56	227	91	205
10	40	262	8	247	20	262	0	262	78	105	50 86	105 160
15	48	262	50 64	247 262	50 68	247 262	4	247	78	86	50 82	77 127
20	0	262	4	220	32	262	0	262	50 78	34 70	50 86	34 64
25	40	247	20	262	50 73	178 262	4	247	78	13	50 82	16 69
30	32	247	36	125	50 76	94 125	50 64	201 247	67 78	10 13	50 68	13 24
35	50 72	147 210	32	106	44	76	32	232	78 89	10 17	18	13

\* Ungerminated nuts from Canton rotted at temperatures of 30° and 35° C. At 25° C. or below 50 per cent of ungerminated embryos were alive. The ungerminated nuts from West Grove rotted at temperatures above 10° C.

\*\* 25 nuts in each test.

† 9 nuts in each test.

†† 22 nuts in each test.

tests do indicate that the observed results are tied in mainly with the material sealing the dehiscent layer and they are not a function of the living embryo.

*Germination tests.* Table V shows the effect of eight different constant temperatures on the germination of intact hickory nuts. The nuts from each of six different trees were tested separately. The most noticeable thing about data in this table is the great variation of the nuts from different trees in germination at the several temperatures. This is probably

due to different genetic constitution, to the stage of ripening at the time the tests were started, and to variation in the number of defective embryos. Considering the number of defective embryos found in hickory nuts, 30° C. gave good germination of the nuts from all six trees. With one exception for 35° and 15° C., and two exceptions for 25° C., the same was true for these three temperatures. Poor germination percentages were shown for three trees at 20° C. Nuts from none of the trees germinated at 1° C. and none from four of the trees at 5° C., while nuts from two of the trees gave good germination at this temperature and nuts from three trees gave good germination at 10° C.

Removing a portion of the shell from the embryo did not solve the delay in germination of the hickory nuts. Experiments with hickory nuts from West Grove showed that they germinate quite readily regardless of whether the shells were opened, cracked, or intact. Thus again the results indicate that maturity or some related condition at harvest time will control the germination of the hickory nut. These results with the West Grove hickory nuts like those with the walnuts show that the embryo is ready to germinate at the higher temperatures but unlike the walnut it is able to do so regardless of the condition of the shell.

The results given in this table are too variable to be of value except for indicating general trends. Extensive experiments ought to be run with a careful determination of the variation in nuts from different trees and the effect of the stage of after-ripening and defective embryos on the percentage of germination at various constant temperatures.

*Growth of hickory nuts in greenhouse.* During the two years of testing, samples of germinating nuts were planted in pots of garden soil and held in the greenhouse for further development. No significant difference was observed in the rate of growth of nuts germinating at 30°, 20°, or 5° C. The plants from the slow-to-germinate 5° C. condition always had deeper green leaves than those from 20° or 30° C. but otherwise showed no difference. Continued growing of plants over a period of a year still gave no indications that there were any other differences in the plants resulting from germinating the embryos at various temperatures.

#### BUTTERNUT

*Breaking pressure experiments.* Breaking pressure experiments with butternuts placed in soil in the same manner as walnuts and hickory nuts showed no great reduction in pressure when held for 120 days at 6°, 11°, and 17° C. and very little difference when held at 23° C. The average breaking pressure was 665 pounds when the nuts were placed in moist storage and for the three lower temperatures it never fell below 600 pounds. At 23° C. it fell to 550 and at 28° C. to 425 pounds. The nuts always broke open cleanly at the dehiscent layer when sufficient pressure was applied.

No germination of intact nuts was obtained at any temperature, 5° to 35° C., during the course of the 120-day experiment.

*Germination tests.* When a section of the shell was removed over the radicle region of the embryo, as was done with the walnut, relatively prompt germination was obtained. The germination at 20°, 25°, 30°, and 35° C. was 17, 17, 50, and 67 per cent respectively in seven days. By the 14th day in moist medium the germinating percentages were 33, 33, 83, and 83 for the temperatures 20°, 25°, 30°, and 35° C. respectively. There was no further increase in germination, except to 67 per cent at 25° C. The remaining opened nuts rotted due no doubt to injuries caused by opening the shell. At the end of 63 days of moist storage 33 per cent of the opened nuts stored at 10° and 15° C. had germinated and no further germinations were observed in these tests. No germination was obtained at 5° C. during the 84-day period of the test. These results are extremely interesting since here is a nut that gives no marked reduction in breaking pressure with storage in moist soil, yet when the shell is opened growth will take place readily. Because of limitations in obtaining first class experimental material with a known history, these tests were not continued the second year.

*Growth of butternuts in greenhouse.* Seedlings produced from the germinating butternuts were planted in the greenhouse. During the course of a year no differences were observed in the growth of the plants from the various temperatures. There was no indication of dwarfish seedlings due to the lack of low temperature pretreatment of the embryos.

## DISCUSSION

The average internal pressure necessary for breaking the shells of the three kinds of nuts used in this research was several times that found by Müller for the hazelnut. In this investigation as in Müller's the intact nuts were held in germinators for long periods to condition them for germination and the measurement of breaking strength was taken in many cases at the time the intact nuts were germinating. The walnut shells in a germinator at 6° C. for 150 days, at which time there were many germinations of intact nuts, had an average breaking strength of 500 to 525 pounds in the different batches. The intact walnuts in the germinators at 11° C. for the same period were germinating and showed an average breaking strength of the shells of 425 pounds. This gives a breaking strength of walnut shells at the time of germination of about 28 to 33 atmospheres. Intact walnuts in germinators for the same time at 17°, 23°, and 28° had considerably weaker shells but no germinations occurred at these temperatures. The breaking strength of the shells of the West Grove intact hickory nuts at the time considerable germinations occurred was at 6° C., 340; at 11° C., 279; and at 28° C., 260 to 300 pounds. The shells of the Canton

nuts showed a somewhat lower breaking strength at the time germination of intact nuts was occurring, 155 to 200 pounds. The pressures for breaking hickory nuts ranged from about 10 to 22 atmospheres. The intact butternuts were kept in germinators only 120 days and no germinations of intact nuts occurred in any temperature during this period but the breaking pressure of the shells at the end of this period even at 28° C. was still 435 pounds or about 28 atmospheres. Müller found the breaking strength of hazelnut shells about 3 atmospheres against walnuts 28 to 33, hickory nuts 10 to 22, and butternuts about 28 atmospheres.

It would seem that the internal water pressure method should be more accurate than the ring method of Müller, for the dehiscent layer may be weakened by sawing the ring out; also the breaking strength in Müller's method had to be calculated from the data on ring strength while the internal water pressure method is a direct measurement. The pressure in the internal water pressure method in this research was applied as thrusts with each stroke of the cylinder. The coats would likely resist greater pressures if the pressure increased very gradually and without thrusts. In these experiments the pressure was increased rather rapidly until the shell split. When the growing embryo splits the shell, the pressure is increased gradually over a long period until the coat splits. This may develop fatigue in the dehiscent tissue and cause the break at a lower pressure. Müller's determinations indicated this. Pressures considerably below quick breaking pressure should be run on intact nuts over long periods to get a measure of the fatigue factor.

The fact that both intact walnuts and butternuts can remain in moist soil at certain temperatures for much more than 100 days without germination, and yet the embryos germinate fairly promptly when the shells over the radicle end are removed, suggests that the mechanical strength of the coat prevents germination. One must not, however, neglect the possibility that the shells may hold in growth inhibiting substances. If this be the case, it does not hold for the walnut at 6° and 11° C. for the intact nuts begin germinating at these temperatures after 90 days. If inhibiting substances account for the failure to germinate at higher temperatures these substances must form faster at high than at low temperatures or they must diffuse out more slowly. The latter is not probable. Further research must be done to answer the growth inhibitor question. The relation between breaking pressure of the coat and growing pressure of the enlarging embryo will have further light thrown upon it when the previously proposed chemical analyses are made of radicle region of embryos of intact walnuts that have been held for long periods in germinators at 6° or 11° C. on one hand and 23° or 28° C. on the other.

It must be recognized that in nature there are factors leading to the weakening of the shells that were not operative or were purposely avoided



in these experiments. It has already been mentioned that the shells of hickory nuts crack at the dehiscent layer if they are allowed to remain on the ground and dry in sunlight after falling from the tree. This probably holds for walnuts and butternuts but the drying is much slower due to indehiscent outer layer of the fruits. No study has been made of the effect of freezing and thawing on the strength of the dehiscent layer. In nature, shell-weakening factors other than those studied in these tests may be more important in removing the shell inhibition to germination than those studied in this research.

Müller found that the breaking strength of the dehiscent layer of the hazelnut shell was about twice as great when the shell was dry as when fully imbibed with water. He also found that when an imbibed shell was again dried the old breaking strength was regained and vice versa. Of course the breaking strength of a given shell or given ring of a shell can be determined only either in the dry or imbibed condition; also there is a great variation in the breaking strength of individual shells, perhaps also different rings from the same shell. This means that a great number of determinations must be made to even out the individual variations. Colloidal materials, such as paper and leather, are much weaker when wet than when dry. This does not justify the conclusion that the same is true of the dehiscent layer of nut shells. Indeed, the measurements on walnut shells mentioned above indicate that the opposite is true; the dehiscent layer of these shells is considerably stronger when wet than when dry.

Müller asserted that the breaking strength of the dehiscent layer of the walnut and other fruit coats in which the dehiscent layer is not cellulosic in nature is not modified by soil organisms. The evidence in this paper shows conclusively that soil organisms are effective in weakening the dehiscent layer of nuts. In water or soil no weakening occurs in absence of organisms and weakening is much slower at low than at high temperatures. We have recognized fully the significance of seed and fruit coats in delaying germination. Apparently we are far from realizing fully the significance of soil organisms (7, 9) in overcoming these coat effects.

While the embryos of these nuts with the shell restriction removed do not germinate speedily, yet 67 per cent germination in the butternut after seven days at the best temperature does not indicate dormancy in the embryos. The embryos of walnuts and hickory nuts showed slower response. It must be remembered that the thin coats surrounding the embryos were intact in all these tests and that these coats may delay germination somewhat. The seeds have fats as the main storage materials and hence need much oxygen to transform the fats to carbohydrates. At least the possible significance of these coats in slowing germination of the embryos should be investigated. Secondary dormancy is often induced in seeds and even in embryos (5, 6, 17) by unfavorable conditions in the

germinator. Although shells of the walnuts prevented the embryo from germinating for at least 150 days at higher temperatures no secondary dormancy was produced in the embryos. The same was true for the butter-nuts at all temperatures for a 120-day period.

As is evident from the discussion above, this research has opened up and defined more problems than it has solved. This is likely to be the case when a new method is applied to an old problem. It is hoped that in the near future further research will bring at least partial solutions of these newly defined problems.

#### SUMMARY

1. Walnuts planted in moist soil at high temperatures, 17°, 23°, and 28° C., showed a reduction in the internal water pressure necessary to break the shell from 627 pounds at harvest time to approximately 275 pounds at the end of 135 days, while walnuts stored at lower temperatures, 6° and 11° C., showed a reduction to 545 and 425 respectively.

2. Embryos of walnuts in intact shells in soil at 6° or 11° C. began to germinate after 90 days and continued to germinate to the end of the 150-day test period. At 17°, 23°, and 28° C. no germinations occurred even after 150 days. The embryos at the two lower temperatures apparently exert enormous pressures against the shells; as much as 28 atmospheres unless fatigue occurs in the dehiscent layer due to ever increasing pressure of the growing embryos.

3. At the higher temperatures the embryos did not exert pressures sufficient to break the shells where the pressure required amounted to about 18 atmospheres.

4. Breaking pressure data show that any collection of walnut and hickory nuts is composed of a mixed population that can be divided into two almost separate groups, even when the nuts are all from a single tree, a high pressure and a low pressure group. Histograms prepared from the data indicate skew distribution curves.

5. As periods in moist soil progressed there was a definite transfer from the high-breaking pressure group into the low-breaking pressure group showing a change in the adhesive quality of the material sealing the dehiscent layer connecting the two halves of the shell. This occurred more rapidly in the high than in the low temperature germinator.

6. The intact green hulls about the walnuts prevented the drop in breaking pressure during the period in moist soil; in fact the pressure increased during storage at 6° C. At higher temperatures where the hulls disintegrated in the soil the pressure dropped in a uniform manner.

7. Sterilizing the shell and holding the nuts in water resulted in no significant change in breaking pressure during 150 days of storage.

8. Chemical analyses of the entire embryos gave no significant changes which accounted for either dormancy or growth of the embryos in intact

shells at any temperature in the germinator. It is suggested that chemical and osmotic studies ought to be made of the radicle region of the embryos after the intact nuts have been in a germinator for a long period at a high and a low temperature.

9. Absorption of water by the walnut embryo takes place at an equal rate whether the shell is open or intact.

10. Removing a portion of the shell over the radicle region allowed germination of the walnut at temperatures ranging from 6° to 28° C. in comparison with no germination of intact walnuts at 17° C. or above and with a slower rate of germination at lower temperatures.

11. Intact walnuts stored three months at 20°, 25°, and 30° C. in moist peat germinated readily when a portion of the shell was removed from the radicle region.

12. Walnut, hickory nut, and butternut seedlings produced from nuts germinated at high temperatures following removal of a portion of the shell do not produce dwarfish seedlings as is the case with certain rosaceous embryos that have not had low temperature treatment.

13. Breaking pressure of hickory nuts was 670 pounds at harvest and the pressure was rapidly reduced by storage in moist soil at all temperatures.

14. When harvested early, the dehiscent layer of the hickory nut shell is so strong that under internal pressure the shell breaks at other regions rather than at the dehiscent layer.

15. Intact hickory nuts germinate readily at high temperatures, 20° to 35° C., but very slowly at low temperatures, 5° to 10° C., and there is a direct relationship between lowering of breaking pressure and germination.

16. When hickory nuts are first placed in 6° C. moist storage there is a reduction in breaking pressure followed by an increase which reaches a maximum before it gradually declines during prolonged storage. This resulted irrespective of the vitality of the embryo.

17. Butternuts kept in moist soil for 120 days show very little reduction in breaking pressure which was 665 pounds at the start of the tests.

18. Butternuts germinate readily when a portion of the shell over the radicle region is removed and the nuts are held in moist peat at 20° to 35° C.

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# POLYMERIC ORGANIC POLYSULPHIDES AS FUNGICIDES AND SPRAY ADJUVANTS<sup>1</sup>

W. D. STEWART AND J. H. STANDEN

## INTRODUCTION

This paper has as its objective the introduction of a new class of spray materials, high polymers of organic polysulphides, characterized by an unusual combination of properties—fungicidal activity and remarkable tenacity of spray residue. These polymers of high sulphur content when sprayed on plants deposit to form translucent, non-phytotoxic, elastic films which are insoluble in water and oil, strongly adhesive for plant surfaces, and highly resistant to removal by rain and weathering.

A new process developed by The B. F. Goodrich Company for the preparation of previously known polymeric organic polysulphides as stable latices of fine particle size made the evaluation of these polymers as fungicides and sprays possible. The latices of polymeric organic polysulphides were found to be fungicidal. Activity is associated with the solid or polymer phase. Toxicity to fungi increases as the sulphur content of the polymer is raised.

Polyethylene pentasulphide, considered the most feasible economically of the group, was tested in the field in 1945. When applied as a preventive spray under seasonal conditions of high and frequent rainfall, latices of this polymer, either alone or in combination with sulphur, gave excellent control of apple scab; sulphur alone at a higher level of concentration than is normally used failed to protect.

Latices of polyethylene pentasulphide are highly stable and hence are well-adapted for application with high pressure spray equipment. They are compatible with oil and with the standard insecticides and fungicides. They confer adhesive properties to spray residues from mixed sprays, promote build-up of spray deposit, and increase the efficiency of the toxicant.

The demonstration of fungicidal activity for huge molecules, such as these, presents the chemist with a new concept in designing more efficient compounds for the control of plant pests. A polymer molecule may be tailored to fit the pest by the choice of toxic groups built into the molecule or attached thereto after synthesis.

The polymeric organic polysulphides are new tools for the plant pathologist. These polymers of high molecular weight offer him labile, active sulphur atoms instead of molecular sulphur as a fungicide. These atomic

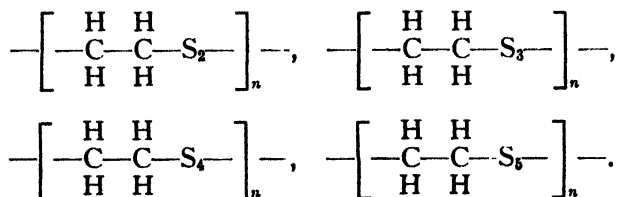
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sulphur groups are appended to a large molecule which, unlike molecular sulphur, does not volatilize in hot weather and injure the plant. Latices of these elastomers are compatible with oil and can be substituted for sulphur as the fungicide in spray schedules requiring summer oil for insect control. They may be utilized in the tropics and other regions of high rainfall where maintenance of spray deposit is difficult, as fungicide and as adhesive for insecticides to secure adequate cover. In the compounding of mixed sprays the rapid build-up of spray residue associated with the presence of the polymer permits reduction in concentration of insecticide without loss in protection.

Polyethylene pentasulphide is prepared by condensing ethylene dichloride and sodium pentasulphide. Raw materials and process are inexpensive. It is a promising fungicide and spray adjuvant.

#### MATERIALS

All polymers were obtained from the laboratories of The B. F. Goodrich Company as lattices with a particle size ranging from one to four microns. A series of polymers was had for the fungicidal studies in which the values for " $S_x$ " in the structural unit  $—[R—S_x]—$  were 2, 3, 4, and 5 for each type of polymer. For example, the polyethylene polysulphides utilized were



The organic grouping "R" of the molecular unit  $—[R—S_x]—$  was varied by condensing formaldehyde, ethylene dichloride, and dichloroethyl ether with sodium polysulphides.

These polymers had received three washings with water by decantation to remove soluble salts. Total solids content ranged from 50 per cent to 60 per cent. Salt content varied from 2 to 4 per cent and the pH from 7.0 to 7.5.

In another series aliquots of a latex of polyethylene pentasulphide were treated with solutions of metallic salts to bind any free thiol, sulphide, or polysulphide groups present. One per cent on the polymer of calcium nitrate, zinc sulphate, neutral lead acetate, copper sulphate, and cobalt chloride respectively was employed. On settling, the polymers were washed five times with tap water by decantation to remove unreacted salt. These preparations bear the symbol of the metal for identification.

Several batches of lattices of polyethylene pentasulphide obtained at

intervals during the spraying season were utilized for the field tests. Physical properties of these polymers were similar to those already described.

Dispersing agents, insecticides, and other fungicides used in spray mixtures were standard materials available on the market.

#### FUNGICIDAL ACTIVITY OF THE POLYMERIC ORGANIC POLYSULPHIDES

Fungicidal activity of the polymers was assessed by the agar plate method and the slide-germination technique. In applying the agar plate technique the polymers were added, after dilution of the latices with tap water to yield desired concentrations, to 12.5 cm. of warm, liquefied, sterile Difco malt extract agar. These were poured into Petri dishes and inoculated by spraying the surface of the agar with aqueous spore suspensions of *Alternaria solani* (Ell. & Mort.) Jones & Grout., Delaware strain, and of *Sclerotinia fructicola* (Wint.) Rehm. The plates were incubated at  $21^{\circ}\text{C.} \pm 1^{\circ}\text{C.}$  Inhibition of growth was ascertained at the end of a three-day period. The same organisms and mode of dilution were used in evaluating the polymers by the slide-germination method (1). The only modification of procedure was the substitution of filtered orange juice for the ultra-filtered juice recommended for stimulating spore germination. The spore suspensions for both procedures were prepared in the same manner by the method described for the slide-germination technique. Tests by both the agar plate and slide-germination methods were duplicated trials. Spore germination of the control series was never less than 95 per cent. Per cent inhibition of spore germination was based on counts of at least 100 spores for each slide. Values indicated were interpolated to the nearest integer of five. All tests were repeated. The values shown in the tables are averages derived from the sum of the counts from four slides or from four plates.

The data in Table I, on the behavior of three different types of polymers of varied sulphur content, show quite clearly that sulphur content of polymers is the dominant factor in the determination of fungicidal activity. Toxicity to the fungi increased as the sulphur content of polymers was raised. Spatial distribution of the sulphur groups within the molecule for separatory units of one, two, or five atoms did not appear to influence fungicidal properties.

Blocking of terminal SH-groups of the polymer molecule with heavy metals, as shown by the results in Table II, did not alter fungicidal behavior. Nor did the presence of copper or other heavy metals known to possess fungicidal properties render the polymers more toxic for the test organisms.

Since the molecules of the polymeric organic polysulphides are of high molecular weight and very inert, any postulates dealing with mechanisms of fungicidal action must of necessity involve consideration of the labile



TABLE I

TOXICITY OF POLYMERIC ORGANIC POLYSULPHIDES FOR *ALTERNARIA SOLANI* AND *SCLEROTINIA FRUCTICOLA* BY THE AGAR PLATE AND SLIDE-GERMINATION METHODS

Polymer	Concn. in p.p.m.	Per cent inhibition			
		Growth		Spore germination	
		<i>A. s.</i>	<i>S. f.</i>	<i>A. s.</i>	<i>S. f.</i>
—[CH <sub>2</sub> S <sub>2</sub> ] <sub>n</sub> —	1200	100	100	0	0
	120	100	0	—	—
	12	0	0	—	—
—[CH <sub>2</sub> S <sub>3</sub> ] <sub>n</sub> —	1000	—	—	0	0
	100	100	100	0	100
	10	0	75	0	0
—[CH <sub>2</sub> S <sub>4</sub> ] <sub>n</sub> —	1000	100	100	100	100
	100	100	100	0	50
	10	0	75	0	0
—[CH <sub>2</sub> S <sub>5</sub> ] <sub>n</sub> —	1000	100	100	100	100
	100	100	100	0	0
	10	0	75	0	0
—[C <sub>2</sub> H <sub>4</sub> S <sub>2</sub> ] <sub>n</sub> —	1000	0	50	0	0
	100	0	0	—	—
	10	75	100	100	50
—[C <sub>2</sub> H <sub>4</sub> S <sub>3</sub> ] <sub>n</sub> —	1000	75	100	100	50
	100	25	95	0	0
	10	0	0	—	—
—[C <sub>2</sub> H <sub>4</sub> S <sub>4</sub> ] <sub>n</sub> —	1000	100	100	100	100
	100	80	100	0	0
	10	50	95	—	—
—[C <sub>2</sub> H <sub>4</sub> S <sub>5</sub> ] <sub>n</sub> —	1000	100	100	100	100
	100	100	100	0	0
	10	75	90	—	—
—[C <sub>2</sub> H <sub>4</sub> OC <sub>2</sub> H <sub>4</sub> S <sub>2</sub> ] <sub>n</sub> —	1000	0	75	0	0
	100	0	0	—	—
	10	100	100	100	100
—[C <sub>2</sub> H <sub>4</sub> OC <sub>2</sub> H <sub>4</sub> S <sub>3</sub> ] <sub>n</sub> —	1000	100	100	100	100
	100	25	95	0	0
	10	0	0	—	—
—[C <sub>2</sub> H <sub>4</sub> OC <sub>2</sub> H <sub>4</sub> S <sub>4</sub> ] <sub>n</sub> —	1000	100	100	100	100
	100	0	75	0	20
	10	0	0	0	0
—[C <sub>2</sub> H <sub>4</sub> OC <sub>2</sub> H <sub>4</sub> S <sub>5</sub> ] <sub>n</sub> —	1000	100	100	100	100
	100	100	0	0	100
	10	100	0	0	0
—[C <sub>2</sub> H <sub>4</sub> OC <sub>2</sub> H <sub>4</sub> S <sub>6</sub> ] <sub>n</sub> —	1000	100	100	100	100
	100	100	0	0	100
	10	100	0	0	0
—[C <sub>2</sub> H <sub>4</sub> OC <sub>2</sub> H <sub>4</sub> S <sub>7</sub> ] <sub>n</sub> —	1000	100	100	100	100
	100	100	0	0	100
	10	100	0	0	0
—[C <sub>2</sub> H <sub>4</sub> OC <sub>2</sub> H <sub>4</sub> S <sub>8</sub> ] <sub>n</sub> —	1000	100	100	100	100
	100	100	0	0	100
	10	100	0	0	0
—[C <sub>2</sub> H <sub>4</sub> OC <sub>2</sub> H <sub>4</sub> S <sub>9</sub> ] <sub>n</sub> —	1000	100	100	100	100
	100	100	0	0	100
	10	100	0	0	0
—[C <sub>2</sub> H <sub>4</sub> OC <sub>2</sub> H <sub>4</sub> S <sub>10</sub> ] <sub>n</sub> —	1000	100	100	100	100
	100	100	0	0	100
	10	100	0	0	0
—[C <sub>2</sub> H <sub>4</sub> OC <sub>2</sub> H <sub>4</sub> S <sub>11</sub> ] <sub>n</sub> —	1000	100	100	100	100
	100	100	0	0	100
	10	100	0	0	0
—[C <sub>2</sub> H <sub>4</sub> OC <sub>2</sub> H <sub>4</sub> S <sub>12</sub> ] <sub>n</sub> —	1000	100	100	100	100
	100	100	0	0	100
	10	100	0	0	0

sulphur atoms along the molecular chain and the possibility of polysulphide formation. Hence it seemed desirable to have data on the fungicidal behavior of the inorganic polysulphide solutions from which the polymers were condensed.

Sodium polysulphide solutions were prepared and the value of "x" in the formula  $\text{Na}_2\text{S}_x$  varied from two to five. Two molar solutions of each polysulphide were made in accordance with the equation of Tartar and Draves (14),  $6\text{NaOH} + (2x + 2)\text{S} \rightarrow 2\text{Na}_2\text{S}_x + \text{Na}_2\text{S}_2\text{O}_3 + 3\text{H}_2\text{O}$ . The reactions were initiated at room temperature using solutions of 40 per cent NaOH in slight excess and sulphur wet with methyl alcohol. Only the heat of reaction was utilized in the preparation of the solutions.

The solutions were stored in sealed glass containers, to prevent loss of polysulphide sulphur by oxidation, until ready to use.

TABLE II

TOXICITY OF POLYETHYLENE PENTASULPHIDE AFTER TREATMENT WITH SOLUTIONS OF HEAVY METAL SALTS, FOR *ALTERNARIA SOLANI* AND *SCLEROTINIA FRUCTICOLA* BY THE AGAR PLATE METHOD

Heavy metal	Polymer concn. in p.p.m.	Per cent control of growth	
		A. s.	S. f.
Cu	1000	100	100
	100	75	50
	10	25	25
Ca*	1000	100	100
	100	100	100
	10	50	0
Co	1000	100	100
	100	100	100
	10	50	50
Zn	1000	100	100
	100	100	100
	10	25	50
Pb	1000	100	100
	100	100	75
	10	50	50
Fe	1000	100	100
	100	100	90
	10	50	25

\* For comparison.

The solutions were diluted with distilled water and tested against *Alternaria solani* and *Sclerotinia fructicola* by the agar plate method and the slide-germination technique.

It is evident, from the data in Table III, that the highly soluble sodium polysulphides are much more toxic for *Sclerotinia fructicola* than for *Alternaria solani*. However, the higher polysulphides are no more toxic than the disulphide. Comparison of the behavior of the sodium polysulphides with that of the polymeric organic polysulphides (Table I) indicates a difference in mechanism of fungicidal action. Activity of the polymers increases with rise in sulphur content. The polymers of high sulphur content

display almost equal toxicity for the two organisms, although less toxic than the sodium polysulphides for *Sclerotinia fructicola*. These polymers are superior to the inorganic polysulphides for the control of *Alternaria solani*.

Aqueous dispersions containing 50 to 60 per cent of polymers were used for dilution in evaluating fungicidal properties of various polymers. This mode of testing yields a value which is the sum of the activity of the solid and aqueous phases. Although the average molecular weight of these

TABLE III

TOXICITY OF SODIUM POLYSULPHIDES FOR *ALTERNARIA SOLANI* AND *SCLEROTINIA FRUCTICOLA* BY THE SLIDE-GERMINATION METHOD USING GLASS SLIDES AND BY THE AGAR PLATE PROCEDURE

Polysulphide	Concn. in p.p.m.	Per cent inhibition			
		Growth		Spore germination	
		<i>A. s.</i>	<i>S. f.</i>	<i>A. s.</i>	<i>S. f.</i>
$\text{Na}_2\text{S} \cdot \text{S}$	1000	50	100	80	100
	100	0	100	0	100
	10	0	100	0	50
	1	0	100	0	0
$\text{Na}_2\text{S} \cdot \text{S}_2$	1000	50	100	100	100
	100	0	100	0	100
	10	0	100	0	100
	1	0	100	0	0
$\text{Na}_2\text{S} \cdot \text{S}_3$	1000	50	100	100	100
	100	0	100	0	100
	10	0	100	0	100
	1	0	100	0	0
$\text{Na}_2\text{S} \cdot \text{S}_4$	1000	50	100	100	100
	100	0	100	0	100
	10	0	100	0	100
	1	0	100	0	0

polymers is known to be very high with consequently extremely low solubilities in water of polymer, the possibility of formation of condensation products of low molecular weight during polymerization with sufficient solubility in water to alter the fungicidal value could not be precluded. Hydrolysis, molecular rearrangement or fragmentation, or oxidation might occur during storage forming soluble products toxic to microorganisms. Information was desired as to the toxicity of the aqueous phase of the latices relative to that of the polymer, and of the diminution or maintenance of toxicity of sera, by renewal from the solid phase, with repeated washings with water.

These concepts were put to experiment. The clear, supernatant liquid from a 50 per cent latex of polyethylene pentasulphide, which had been in

storage six months, was removed and tested for fungicidal activity. The polymer was then shaken vigorously with three volumes of water, allowed to settle for 16 days, and the supernatant liquid removed by decantation. This fraction contained some extremely fine particles of polymer which failed to settle out. It was discarded, and the polymer again taken up in three volumes of water, shaken vigorously, and allowed to settle for eight days. The clear supernatant liquid was removed by decantation and examined for fungicidal activity. The polymer was again washed as above, and the clear supernatant liquid removed for fungicidal assay. Fungicidal activity of these fractions, as measured by the slide-germination and agar plate techniques, was slight or lacking even at dilutions as low as 1:100. Toxicity is associated with the solid or polymer phase.

#### LATICES OF POLYETHYLENE PENTASULPHIDE AS PREVENTIVE SPRAYS FOR THE CONTROL OF APPLE SCAB

A fifteen-year-old apple orchard located within ten miles of the weather station at Pough-waugh-on-onk near New Paltz, New York, was selected for field tests in 1945. This three-acre orchard was planted with Rome, McIntosh, Red Delicious, and Golden Delicious. The trees were heavily infected with scab, *Venturia inaequalis* (Cke.) Wint., in 1944. Sources of primary inoculum were excellent.

The sprays were applied with a modern spray unit, equipped with a single gun, at 350 to 400 lbs. pressure. Sulphur, either Koppers wettable or Corona micronized, was dispersed with the spray nozzle in a five-gallon milk can and then screened into the spray tank. The polyethylene pentasulphide latex was poured directly into the filled spray tank. Seven sprays were applied at approximately two-week intervals from the 8th of April to the 4th of July. Three trees of each variety or 12 individuals were used for each treatment. Polyethylene pentasulphide was sprayed at dosages of 2, 4, and 8 lb. per 100 gal. water. Sulphur at a high concentration, 10 lb. per 100 gal. water, to secure maximum protection, was used as the standard for comparison. Polyethylene pentasulphide at 2 lb. per 100 gal. water was applied in combination with sulphur, 10 lb. per 100 gal. water, to determine compatibility, and behavior as an adhesive for sulphur.

Temperature and moisture conditions were ideal for infection throughout most of the spraying season and the unsprayed trees of the more susceptible varieties, Rome and McIntosh, were beginning to defoliate by the middle of June. An abnormal season led to an early break of buds, the trees blossoming in April at least three weeks ahead of the normal bloom. Weather was cool and rain fell at intervals of about 48 hours (see Table IV) throughout May, June, and July. Precipitation for May was 7.94 inches, for June 5.62 inches, and for July 8.83 inches. Only fungicides yielding unusually adhesive spray deposits could withstand the almost constant

elution by rain and protect the foliage against the scab organism. Even with these it was difficult to cover new foliage as it formed, so rapid was infection. Sprays were applied April 8, 17, 26, May 14, 31, June 21, and July 4, 1945.

Lesion counts on foliage were the index of control. Data on fruit were not feasible since trees in the lower section of the orchard were denuded of fruit by frost shortly after full bloom. Ten branches at the same level around the tree were selected at random on each of the three trees for each variety and the ratio of infected to clean leaves recorded. This involved the counting of about 1500 leaves for each variety for each treat-

TABLE IV  
RAINFALL IN INCHES AT POUGH-WAUGH-ON-ONK WEATHER STATION,  
NEAR NEW PALTZ, N. Y.

April	May	June	July
3—0.69	1—0.62	2—0.27	2—0.36
17—0.03	2—0.08	4—0.25	3—0.09
18—0.28	4—1.35	5—0.23	6—0.54
21—0.06	5—0.20	6—0.02	9—0.06
25—0.80	8—0.31	10—0.12	15—1.69
26—0.60	10—0.98	11—0.09	16—0.01
27—0.30	11—0.09	13—0.02	17—0.08
30—0.07	13—0.43	16—1.24	18—1.07
	16—0.67	17—0.02	19—0.78
	17—0.18	19—0.09	21—0.01
	18—0.58	20—1.16	22—0.65
	19—1.56	22—1.19	23—0.67
	22—0.21	26—1.65	26—1.06
	27—0.46	28—0.20	27—0.52
	28—0.05	29—0.07	29—1.24
	29—0.12		
	30—0.05		
2.83	7.94	5.62	8.83

ment. On the 25th of June when readings were taken, unsprayed trees were badly damaged by the scab fungus; the foliage had assumed the "suede-finish" characteristic of systemic infection of leaves at the periphery of the tree and had begun to abscise.

Degree of control by the sprays applied for the three most susceptible varieties is indicated in Table V. Sulphur at a much higher concentration than is normally used failed to protect. This was due to rapid removal of spray deposit by rain. Polyethylene pentasulphide even at concentrations as low as 0.25 per cent, gave satisfactory control of scab in spite of a rainfall averaging more than seven inches a month over a period of three months. This rainfall compares quite favorably with that of the tropics. There was little difference in effectiveness of the other sprays. The values indicate almost perfect protection in that the little infection present consisted chiefly of lesions developing on unprotected new growth formed

in the intervals between sprays. None of the trees of any of the varieties displayed any evidence of damage. Color of foliage was excellent.

In an adjoining commercial orchard of several acres of mature Baldwins and McIntosh, owned by Mr. Carleton Van de Water, dusting with sulphur was practised for the control of scab. Rapid removal of sulphur by the frequent rains the last week of April and the first week of May combined with ideal conditions for spore germination resulted in a heavy infection of foliage with scab. On May 6 polyethylene pentasulphide at 1 lb. per 100 gal. water was applied with lime-sulphur at 1 gal. per 100 gal. of water and arsenical (5 lb. lime and 5 lb. lead arsenate per 100 gal. water) to destroy the lesions. The scab was eradicated without apparent injury

TABLE V  
CONTROL OF APPLE SCAB IN FIELD TESTS AT NEW PALTZ, NEW YORK.  
READINGS TAKEN JUNE 25, 1945

Treatment	Per cent infected leaves		
	Red Delicious	McIntosh	Rome
Control—unsprayed	71*	Defoliating	Defoliating
10 lb. Sulphur/100 gal.	9.0	42*	15
2 lb. Polyethylene pentasulphide/100 gal.	0.4	7.0	1.0
4 lb. Polyethylene pentasulphide/100 gal.	0.9	1.7	0.4
8 lb. Polyethylene pentasulphide/100 gal.	0.7	2.0	0.4
2 lb. Polyethylene pentasulphide/100 gal. + 10 lb. sulphur/100 gal.	0.4	0.9	1.0

\* Multiple lesions. Leaves at tip of shoots frequently covered with fungus, curling, ready for abscission.

to the foliage. Two gallons of lime-sulphur per 100 gal. water which is normally required for "burning-out" scab lesions endangers the foliage.

Later in the season, June 5, polyethylene pentasulphide was again used with lime-sulphur at the same concentration, in a mixed insecticidal spray of nicotine sulphate and arsenical for the eradication of scab in another unit of the same orchard. Here, too, the lesions were destroyed without apparent injury to foliage.

#### TENACITY OF DEPOSIT OF SPRAYS CONTAINING POLYETHYLENE PENTASULPHIDE

The build-up of spray deposit with the use of sprays containing polyethylene pentasulphide under conditions of high rainfall is remarkable. When used alone the spray residue from polyethylene pentasulphide is translucent and difficult to see (leaf on left, Fig. 1 B). In combination with sulphur the build-up of spray deposit is readily apparent (middle leaf, Fig. 1 B).

The last application of sprays for the control of apple scab was made the fourth of July. After six weeks' leaching with 9.7 inches of intermittent

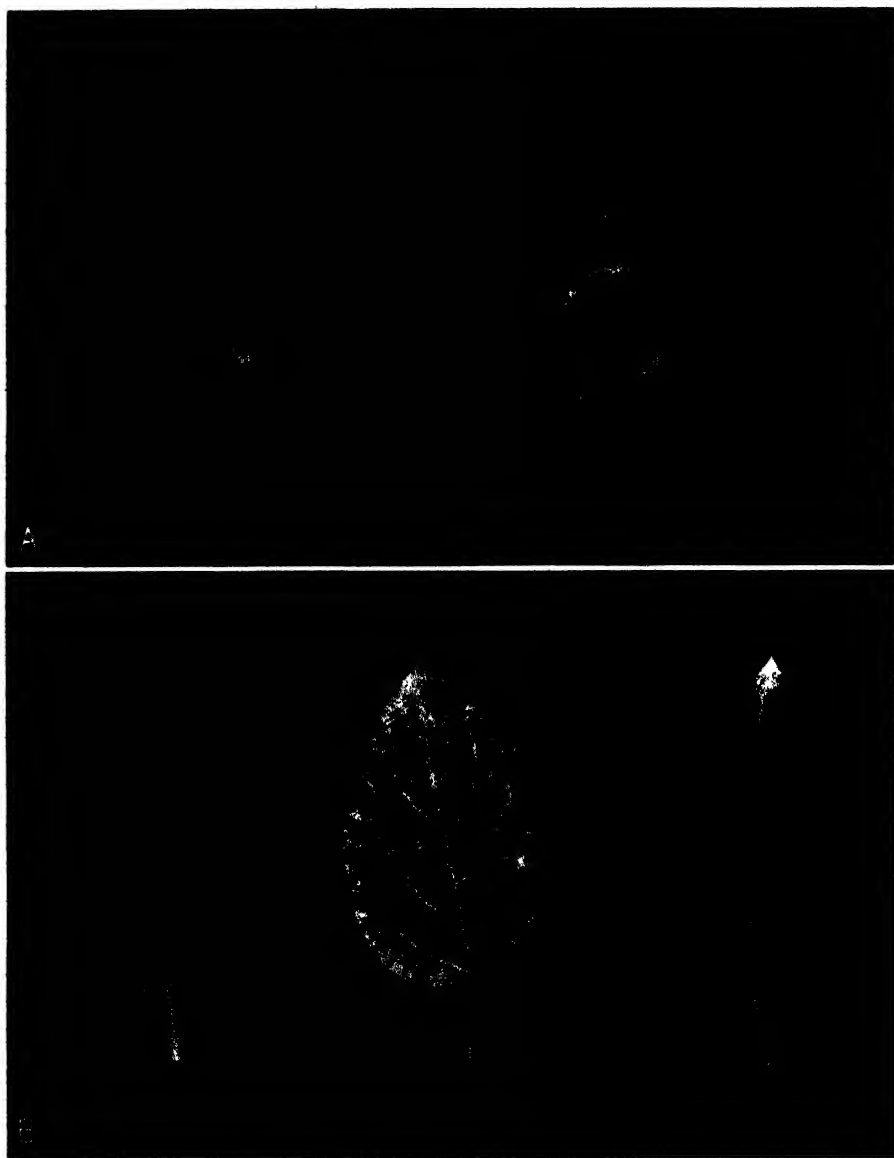


FIGURE 1. A. (Left) Arsenical injury, Baldwin apples sprayed with 0.25% polyethylene pentasulphide, 4 lb. lead arsenate per 100 gal., 4 lb. lime per 100 gal. (Right) Baldwin apples sprayed with 0.25% polyethylene pentasulphide, 2 lb. lead arsenate, 2 lb. lime. B. Spray residue after 6 weeks' weathering (9.7 inches rain). (Left) Sprayed with 0.25% polyethylene pentasulphide, translucent spray deposit not visible; (Middle) sprayed with 0.25% polyethylene pentasulphide and 10 lb. sulphur/100 gal., spray deposit visible; (Right) sprayed with 10 lb. sulphur per 100 gal., spray deposit removed by rain.

rains the foliage of the trees sprayed with sulphur and polyethylene pentasulphide was still grey with spray deposit; those sprayed with sulphur alone were free of deposit except at the tips of occasional leaves. Photographs of the leaves at the end of the six weeks' weathering period are shown in Figure 1 B.

More concrete evidence of this resistance to weathering of spray deposits was sought. Sulphur values for the leaves at the end of the six weeks' leaching would permit comparisons. Mature leaves of comparable age were collected from McIntosh trees, and the area of the leaves determined by a planimeter from outlines of the leaves traced on paper. Twenty-five leaves were obtained from trees of each experimental group. The leaves were then dried and sulphur content ascertained by the method of Parks *et al.* (4). Relative values for each spray are shown in Table VI.

TABLE VI

TENACITY OF SPRAY DEPOSITS OF SPRAYS CONTAINING POLYETHYLENE PENTASULPHIDE AS INDICATED BY SULPHUR CONTENT OF APPLE LEAVES AFTER SIX WEEKS' WEATHERING (RAINFALL = 9.7 INCHES)

Spray	% Sulphur, dry wt. basis	Mgm. S/sq. in. leaf surface
Sulphur only—10 lb./100 gal.	0.31	0.19
Polyethylene pentasulphide only— 2 lb./100 gal.	0.73	0.54
Polyethylene pentasulphide only— 8 lb./100 gal.	1.36	0.85
Polyethylene pentasulphide 2 lb./100 gal. + sulphur 10 lb./100 gal.	1.84	1.09

The high sulphur values for apple leaves sprayed with polyethylene polysulphide relative to that of leaves sprayed with sulphur are convincing evidence of the tenacity and resistance to weathering of spray deposits of this polymer. The analyses also show that the polymer is an excellent adhesive for sulphur.

#### FIELD TRIALS WITH POLYETHYLENE PENTASULPHIDE FOR PHYTOTOXICITY AND COMPATIBILITY AS SPRAY ADJUVANT

The rose (*Rosa* sp.) plant, being highly susceptible to injury by chemicals, was selected for phytotoxic tests under field conditions. Five applications of sprays were made at weekly intervals in June and July, 1944, at a concentration of one per cent of a series of polyethylene pentasulphides, including the six latices treated with metallic salts previously described (Table II), to rambler rose plants heavily infected with powdery mildew, *Sphaerotheca humuli* (D.C.) Burr. All controlled the mildew; none produced any symptoms of injury. There was no discoloration of foliage or leaf fall. Appearance of treated and untreated foliage is shown in Figure 2.

Particle size and shape of one of the polymers treated with heavy metal salts are indicated in Figure 3.



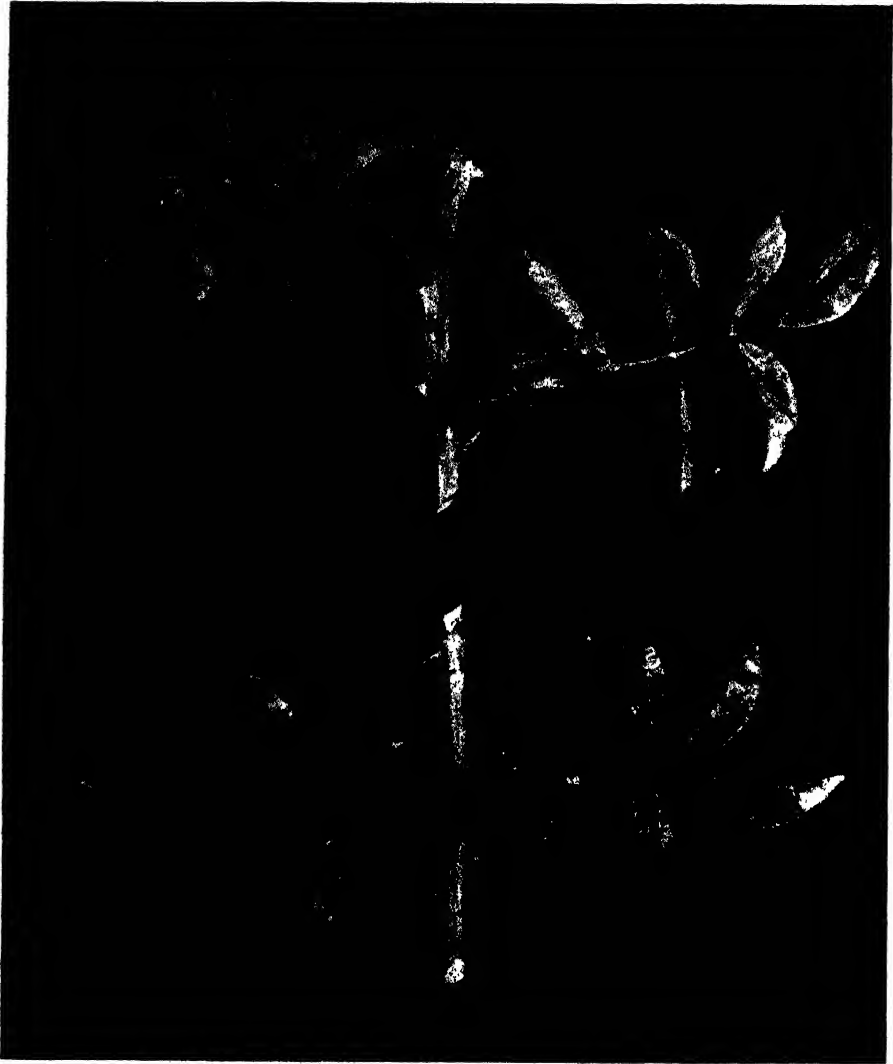


FIGURE 2. Control of powdery mildew on roses. (Top) Unsprayed; (bottom) sprayed with 1% polyethylene pentasulphide.

No evidence of phytotoxicity was had on terminating a spray schedule of five sprays at intervals of approximately ten days at a concentration of 0.25 per cent polyethylene pentasulphide applied to the following plants in the field: potato (*Solanum tuberosum* L. var. Green Mountain), tomato (*Lycopersicon esculentum* Mill. var. Bonny Best), eggplant (*Solanum melongena* L. var. Burpee's Black Beauty), bean (*Phaseolus vulgaris* L. var. Surecrop Stringless Wax), beet (*Beta vulgaris* L. var. Detroit Dark

Red), turnip (*Brassica rapa* L. var. Purple-top White Globe), and onion (*Allium cepa* L. var. Southport Yellow Globe).

Since spray deposits of polyethylene pentasulphide are characterized by unusual tenacity and resistance to weathering and since the polymer imparts this property to other materials on admixing, it was combined and

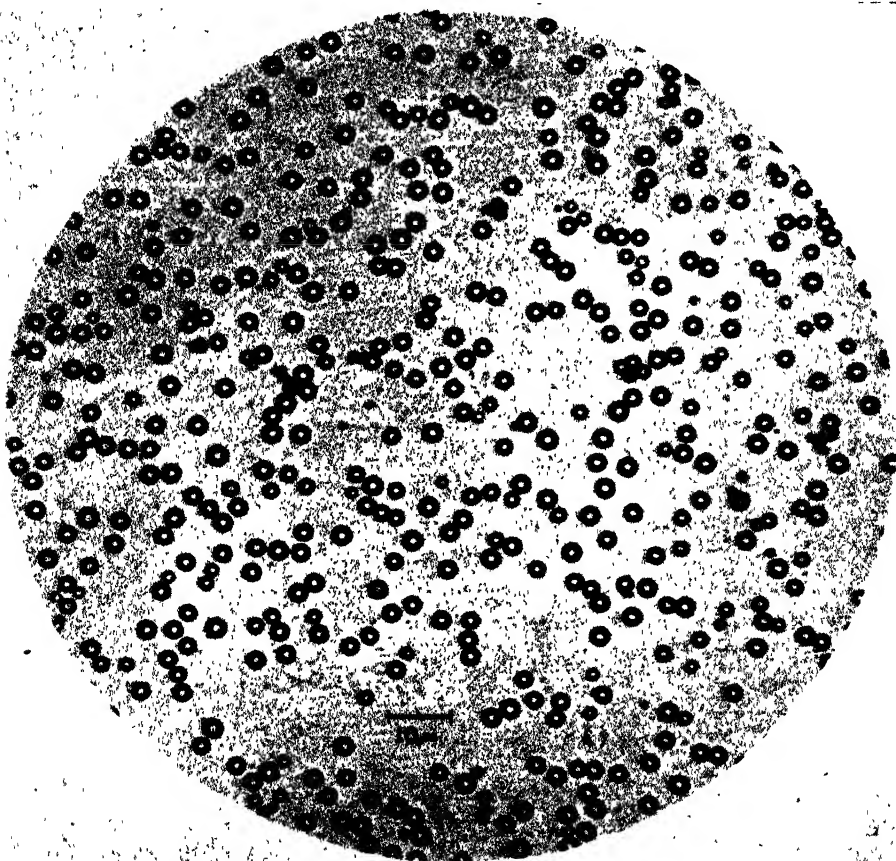


FIGURE 3. Particles—polyethylene pentasulphide latex treated with calcium nitrate, 1% on the polymer.  $\times 768$ .

sprayed with standard insecticides in modern, high pressure equipment to determine physical compatibility. These sprays, containing 0.25 per cent of polymer, were tank-mixed and applied in the same apple orchards and with the spray unit described for the apple scab experiments. Nicotine sulphate (Blackleaf 40), lead arsenate, and lime, summer oil (Ortho K), or mixtures of oil and nicotine at standard dosage were compatible with the polymer dispersion. No flocculation occurred and no difficulty was had

on spraying at 300 to 400 lb. pressure. Dispersing and emulsifying agents such as Goulac (lignin sulphite, etc.), blood albumin, calcium caseinate, Vatsol O. T., sodium alkyl sulphate (Aquarex D), or alkyl aryl sulphonates (Nacconals) were also compatible with the polymer forming stable dispersions and emulsions.

Polyethylene pentasulphide enhanced the activity of nicotine sulphate (Blackleaf 40) against the green aphid, *Aphis pomi* De Geer, of apple. Apple trees of the variety Red Delicious in the experimental orchard were found to be heavily infested with green aphids on March 30, 1945. Leaf buds were just beginning to unfold. Nine trees were sprayed with nicotine sulphate (Blackleaf 40) using 0.75 pint nicotine sulphate per 100 gal. water and nine trees with the same amount of nicotine sulphate and 0.5 per cent polyethylene pentasulphide. Temperature at time of spraying was 65° to 70° F. After 48 hours counts were made of living and dead aphids, using 75 infested buds selected at random on the nine trees of each treatment for the counts. Nicotine sulphate alone gave a kill of 60.5 per cent; that of the nicotine sulphate plus polymer was 99.2 per cent.

Polyethylene pentasulphide at 0.25 per cent was used with lead arsenate to improve adhesion of spray deposit and subsequent build-up of toxicant in the control of codling moth (*Carpocopsa pomonella* Linné) of apples. A minimum of seven trees was selected for each treatment. The varieties sprayed were Golden Delicious, Red Delicious, McIntosh, Baldwin, and Northern Spy. Five sprays were applied for the first brood and two covers for the second brood.

The dosage of lead arsenate was 2 lb. per 100 gal. and 4 lb. per 100 gal. Equal parts of lime (slaked) were added with the arsenate as "safener." Spraying with 4 lb. per 100 gal. of lead arsenate mixture in combination with the polyethylene pentasulphide was discontinued after the fourth cover to avoid serious injury to the trees. Build-up of spray deposit was rapid in spite of heavy rainfall. Ninety per cent of the apples on the Baldwin trees were splitting (see Fig. 1 A), and the remainder was badly russeted and stunted. Foliage was seriously damaged. There were no stings and no entries by larvae of the codling moth. Occasional apples only of Delicious, McIntosh, and Northern Spy bore symptoms of injury evinced as russetting or splitting of fruit. Fruit and foliage of all these varieties sprayed with 2 lb. lead arsenate per 100 gal. and polyethylene pentasulphide appeared normal throughout the spraying season. Less than 4 per cent of the fruit was wormy. This compares favorably with the 8 to 10 per cent entries in the adjoining commercial orchard sprayed with 3 lb. lead arsenate per 100 gal. Unsprayed controls averaged 32 per cent wormy fruit.

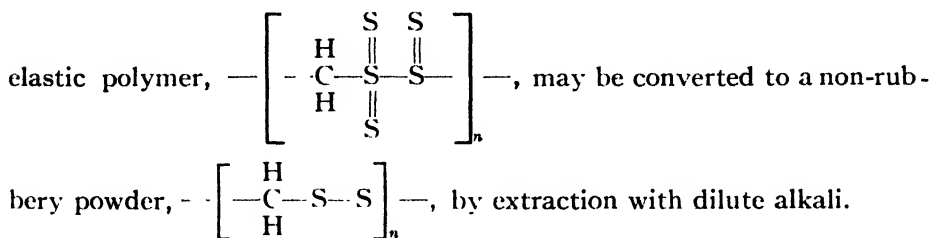
#### DISCUSSION OF RESULTS

Probably the chief deterrent in the past to an evaluation of the polymeric organic polysulphides as spray materials has been inability to pre-

pare dispersions of fine particle size and adequate stability suitable for application to plants. The polymers were known before the twentieth century. In 1840 Löwig and Weidmann (3, p. 126) prepared potassium trisulphide in alcohol and reacted this with ethylene dichloride to obtain an insoluble rubbery substance which on analysis was found to have three atoms of sulphur to two of carbon. Their possibilities as elastomers were recognized by Baer (2) of Switzerland and Patrick (5, 6) of the United States. Patrick exploited a variety of these polysulphides under the trade name of "Thiokol" (7, 8, 9, 10, 11, 12, 13).

A process discovered by The B. F. Goodrich Company permits control of particle size in the preparation of highly stable, finely dispersed, non-phytotoxic latices of polymeric organic polysulphides adapted for utilization as spray materials for plants. Although latices with a range in particle size below a micron may be made, these are impractical for washing by decantation. Latices with particles of one to four microns are better adapted for processing; these were supplied for evaluation in the laboratory.

Latices of three types of polymers were utilized to secure a wide range in sulphur content of polymer and variable inert non-sulphur links in the polymer molecule. The predominant reactive groups are the sulphur atoms which are appended as side chains along the polymer molecule. These are readily removed with desulphurizing agents such as mild alkali, leaving only the highly stable disulphide linkage. For example the rubber-like,



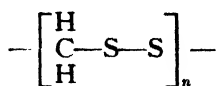
Fungicidal activity is associated with the presence and number of these labile sulphur atoms (Table I) of the polymer. The aqueous phase is almost lacking in toxicity. Blocking of terminal SH-groups with heavy metals did not alter fungicidal activity (Table II). The mechanism of action of the inorganic polysulphides,  $\text{Na}_2\text{S}_x$ , and of the polymeric organic polysulphides appears to differ. The inorganic polysulphides are much more toxic for *Sclerotinia fructicola* than for *Alternaria solani* but toxicity of the polymeric organic polysulphides for the two organisms is similar. The "inert" polymers of high sulphur content are more potent against *Alternaria solani* than the highly soluble inorganic polysulphides. Toxicity of the polymeric organic polysulphides for the two organisms increases with increase in sulphur content of polymer; toxicity of the various inorganic

polysulphides does not differ from disulphide to pentasulphide (see Tables I, II, and III).

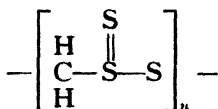
Polyethylene pentasulphide at 2 lb. polymer per 100 gal. was much more effective for the control of apple scab than sulphur at 10 lb. per 100 gal. (Table V) under seasonal conditions of high and frequent rainfall and temperatures very favorable to infection by the pathogen. Sulphur was applied at a much higher level than generally practised to obtain maximum protection by this fungicide.

Tenacity and resistance to weathering of spray deposit are outstanding characteristics of the polyethylene pentasulphide. The polymer particles when sprayed on plants form translucent rubbery films on drying. These almost invisible films are strongly adhesive for leaf surfaces and impart this property to other spray materials when added to spray mixtures. When added to sulphur build-up of visible deposit is rapid, even under conditions of frequent and heavy rainfall. This is shown quite clearly in Figure 1 B and Table VI.

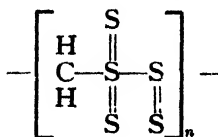
Tenacity of spray residues of the polymeric organic polysulphides and rubber-like properties of polymer are conjoined. Elastic properties of these polymers are regulated both by the number of non-sulphur atoms separating the sulphur groups within the molecule and by the number of sulphur atoms linked to the non-sulphur groups. The larger the number of non-sulphur atoms separating the sulphur groups within the molecule the more rubber-like are the properties of the polymer, and the greater the number of sulphur atoms linking the non-sulphur groups the more rubbery becomes the polymer. For example



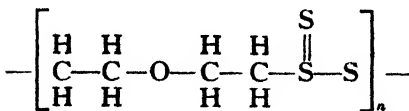
a powder



a powder



rubber-like polymer



rubber-like polymer

Fortunately, increase in sulphur content of polymer is linked with increase in fungicidal activity, elastic properties, and tenacity of spray deposit.

No symptoms of phytotoxicity were observed on any of the many

species of plants sprayed with polyethylene pentasulphides under field conditions. Even the rose plant which was sprayed with a series of polymers at concentrations as high as 1 per cent was not injured. Appearance of foliage was excellent; leaves slightly infected with mildew regained their color after spraying with latices of these polymers at 1 per cent (Fig. 2). Nor was there any evidence of injury on the trees of the four varieties of apple which received seven sprays of 1 per cent polyethylene pentasulphide during the season.

Physical compatibility of latices of the polymeric organic polysulphides with standard insecticides, fungicides, and ancillary spray materials is excellent when tank-mixed. Only one precaution must be observed when mixing; the latex can not be added to dusts before wetting, or to alcoholic solutions and similar dehydrating agents before dilution without loss of stability. When admixed with insecticides and other fungicides, polyethylene polysulphides enhance activity. This is attributed to build-up of spray deposits which are highly resistant to removal by rain. Should the material admixed with the polyethylene polysulphide be slightly toxic to plants this toxicity is greatly augmented as spray residues increase. Spray deposits of lead arsenate, for example, when applied with polyethylene polysulphide at 4 lb. per 100 gal. are so adherent that foliage and fruit may be seriously damaged with only a few sprays (Fig. 1). Smaller dosage of toxicant must be used; these appear to give satisfactory control of pests.

Hopperstead<sup>2</sup> in experiments on the control of apple scab in Delaware in 1945 used polyethylene pentasulphide in combination with Bordeaux, Isothan Q 15, and Fermate. The polymer was compatible with these fungicides and enhanced their effectiveness in the control of scab. The most effective combination was polyethylene polysulphide and Isothan Q 15. The polymer alone at a concentration of 0.25 per cent gave better control than Fermate at a dosage of 1 lb. per 100 gal. water.

The polymer was also applied in combination with insecticides for the control of the codling moth. Polyethylene pentasulphide was compatible with oil, nicotine sulphate, Fixed nicotine-BL155, lime, and DDT. It enhanced the activity of these insecticides.

The spherical particles of the latices are highly stable and give an excellent performance in high pressure spray equipment. They are compatible with oil; the particles do not swell in oil but aid in its dispersion.

#### SUMMARY AND CONCLUSIONS

Polymeric organic polysulphides of high molecular weight can be made as stable, finely-divided, aqueous dispersions with a range in particle size of one to four microns. These are especially suitable for use as spray materials.

<sup>2</sup> Unpublished results, S. L. Hopperstead, Associate Research Professor, Department of Plant Pathology, University of Delaware, Newark, Delaware.

When sprayed on plants these latices dehydrate to form rubbery, non-phytotoxic films insoluble in water and oil, of unusual tenacity and resistance to weathering. On admixing with other spray materials tenacity and resistance to weathering are imparted to the spray deposit.

The polymers were evaluated as fungicides by standard laboratory procedures against *Sclerotinia fructicola* and *Alternaria solani*. Polymeric organic polysulphides are fungicidal. Toxicity to fungi increases with increase in sulphur content of polymer. Activity is associated with the solid or polymer phase of the latices; the aqueous phase is almost non-toxic.

Polyethylene pentasulphides are considered the most feasible for commercial use. Latices of these polymers were tested in 1945 in the field using modern high pressure spray equipment. The polymers were more effective at 2 lb. per 100 gal. as a protective spray for the control of apple scab than micronized sulphur at 10 lb. per 100 gal. under conditions of high and frequent rainfall.

Latices of this polymer when applied in mixed sprays enhanced the activity of toxicants. Build-up of spray deposits was rapid even with high and frequent rainfall. Physical compatibility with standard fungicides and insecticides, oil included, was excellent.

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# REPORT ON SOME MISCELLANEOUS METHYLENEDIOXY-PHENYL COMPOUNDS TESTED FOR SYNERGISM WITH PYRETHRUM IN FLY SPRAYS

EDWARD A. PRILL AND MARTIN E. SYNERHOLM

A number of classes of effective insecticidal compounds containing the methylenedioxyphenyl group in their structures have been synthesized and tested in this laboratory. Most of the data on these compounds have appeared in publications (1, 2, 5, 6, 7) wherein the particular classes of compounds are discussed and literature references to other insecticidally active compounds containing the group are cited.

A general characteristic of the effective methylenedioxyphenyl compounds is their ability to exhibit synergistic action with pyrethrum. In addition to their synergistic activity, some of the compounds possess considerable paralyzing and killing ability toward houseflies even without added pyrethrum. Outstanding among these are piperine (2, 7), which is the principal alkaloid of black pepper (*Piper nigrum* L.), and other related N-substituted amides of piperic acid (2, 7).

The present paper is a report on some miscellaneous methylenedioxyphenyl compounds and deals, for the most part, with those found relatively inactive toward houseflies in the screening tests incidental to the search for active substances. Besides being a matter of record, the report of relatively inactive compounds shows that the presence of a methylenedioxyphenyl group in the structure of a compound is not a sufficient condition for insecticidal activity or for synergistic activity with pyrethrum. A few of the compounds included show moderate activity.

Many of the compounds reported herein are known substances; a few of these are available commercially; others were prepared according to directions given in the literature. Some new compounds were prepared from piperonal by reactions which are general for aromatic aldehydes. The new compounds were not characterized as it was felt that these were not sufficiently active to warrant the attention. However, their identities were reasonably certain from the methods of synthesis used.

The spray solutions were prepared by dissolving in a small volume of acetone (usually 10 ml.) a quantity of the material necessary to give the desired concentration when diluted to 100 ml. with "Deo-base." In a few cases, where it was difficult to dissolve the chemical, a larger amount of acetone was used. The last four compounds in Table I were dissolved in pure ethyl oxalate because of their low solubilities in most other common solvents. In each case 0.025 g. of pyrethrins was added per 100 ml. of solu-



TABLE I  
TOXICITIES TOWARD HOUSEFLIES OF METHYLENEDIOXYPHENYL COMPOUNDS  
WITH ADDED PYRETHRINS

Concentration of Pyrethrins: 0.025 g. per 100 ml. of Solution

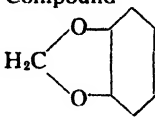
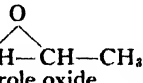
Compound where R is 	Compound, g. per 100 ml.	% Kill (24 hr.)	O.T.I. % kill (24 hr.)
Check (0.025 g. pyrethrins per 100 ml. only)	0 .	19	46
R—CHO Piperonal	4.0	3	28
R—CH <sub>2</sub> —CH=CH <sub>2</sub> Safrole	2.0	20	40
R—CH=CH—CH <sub>3</sub> Isosafrole	2.0	21	46
R—CH=CH—CH <sub>2</sub> ·C <sub>6</sub> H <sub>4</sub> (NO <sub>2</sub> ) <sub>2</sub> OH Isosafrole picrate	0.5	21	44
R—C≡C—CH <sub>3</sub> 3,4-Methylenedioxyphenyl methyl acetylene	1.4	22	45
R—CH <sub>2</sub> —CHBr—CH <sub>2</sub> Br Safrole dibromide	2.0	59	73
R—CHBr—CHBr—CH <sub>3</sub> Isosafrole dibromide	2.0	62	50
 Isosafrole oxide	2.0	62	50
R—CH <sub>2</sub> COCH <sub>3</sub> Piperonyl methyl ketone	2.0	44	60
R—CH(OH)CHBr—CH <sub>3</sub> $\alpha$ -Hydroxy- $\beta$ -bromo-dihydroisosafole	2.0	47	50
R—CH(OC <sub>2</sub> H <sub>5</sub> )CHBr—CH <sub>3</sub> $\alpha$ -Ethoxy- $\beta$ -bromo-dihydroisosafole	1.0 2.0	60 80	55 51
R—CH(O— <i>n</i> -C <sub>4</sub> H <sub>9</sub> )—CHBrCH <sub>3</sub> $\alpha$ - <i>n</i> -Butoxy- $\beta$ -bromo-dihydroisosafole	2.0	78	51
CH <sub>2</sub> O <sub>2</sub> C <sub>6</sub> HBr <sub>2</sub> —CH(OC <sub>2</sub> H <sub>5</sub> )—CHBr—CH <sub>3</sub> $\alpha$ -Ethoxy- $\beta$ -bromo-dihydrodibromoisosafole	2.0	53	55
CH <sub>2</sub> O <sub>2</sub> C <sub>6</sub> HBr <sub>2</sub> —CH(O— <i>n</i> -C <sub>4</sub> H <sub>9</sub> )—CHBr—CH <sub>3</sub> $\alpha$ - <i>n</i> -Butoxy- $\beta$ -bromo-dihydrodibromoisosafole	2.0	48	53
R—CH <sub>2</sub> OH Piperonyl alcohol	1.0	18	40
R—CH <sub>2</sub> Br Piperonyl bromide	1.0	34	56

TABLE I (Continued)

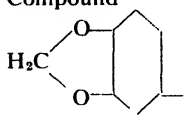
Compound where R is 	Compound, g. per 100 ml.	% Kill (24 hr.)	O.T.I. % kill (24 hr.)
R—CN Piperonylonitrile	1.0	19	40
R—CH=N—OH Piperonaldoxime	2.0	41	39
R—CH=CH—CHO Piperonalacetaldehyde	1.0	20	52
R—CH=C( <i>n</i> -C <sub>4</sub> H <sub>9</sub> )—CHO $\alpha$ -Piperonalhexaldehyde	2.0	79	40
R—CH=CH—NO <sub>2</sub> Piperonalnitromethane	0.5	29	47
R—CH=CH—C <sub>6</sub> H <sub>5</sub> 3,4-Methylenedioxy stilbene	1.0	30	26
R—CH=CH-2,4-(NO <sub>2</sub> ) <sub>2</sub> C <sub>6</sub> H <sub>3</sub> 3,4-Methylenedioxy-2',4'-dinitrostilbene	1.0	23	51
R—CH(OH)—CCl <sub>3</sub> 3,4-Methylenedioxyphenyl trichloromethylcarbinol	0.5	42	44
R—CH=N—C <sub>6</sub> H <sub>5</sub> Piperonalanil	0.5	13	29
R—CH=N— <i>o</i> -ClC <sub>6</sub> H <sub>4</sub> Piperonal <i>o</i> -chloroanil	0.5	19	29
R—CH=N—2,4-(CH <sub>3</sub> ) <sub>2</sub> C <sub>6</sub> H <sub>3</sub> Piperonal 2,4-dimethylanil	0.5	66	47
R—CH=N—CH(CH <sub>2</sub> ) <sub>5</sub> Schiff's base of piperonal and cyclohexylamine	0.5	16	29
R—CH=N—O—CO—NHC <sub>6</sub> H <sub>5</sub> Piperonaldoxime N-phenylcarbamate	1.0	24	43
R—CH=N—O—CH(OH)CBr <sub>3</sub> Piperonaldoxime-bromal	0.5	15	45
R—CH=N—O—CH(O—CO—NHC <sub>6</sub> H <sub>5</sub> )CBr <sub>3</sub> Piperonaldoxime-bromal N-phenylcarbamate	0.5	20	45
R—CH=N—C <sub>6</sub> H <sub>4</sub> CON( <i>n</i> -C <sub>4</sub> H <sub>9</sub> ) <sub>2</sub> Piperonalanil of N-di- <i>n</i> -butyl <i>p</i> -aminobenzamide	1.0	34	51
R—CO <sub>2</sub> — <i>p</i> -CH <sub>3</sub> C <sub>6</sub> H <sub>4</sub> <i>p</i> -Tolyl piperonylate	1.0	38	50
R—CO <sub>2</sub> —2,4,6—Cl <sub>3</sub> C <sub>6</sub> H <sub>2</sub> 2,4,6-Trichlorophenyl piperonylate	0.5	34	51
R—CO <sub>2</sub> — <i>o</i> -ClC <sub>6</sub> H <sub>4</sub> <i>o</i> -Chlorophenyl piperonylate	0.5	19	56

TABLE I (Continued)

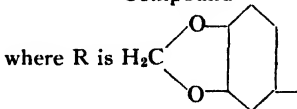
Compound where R is 	Compound, g. per 100 ml.	% Kill (24 hr.)	O.T.I. % kill (24 hr.)
R—CH=CH—CO <sub>2</sub> C <sub>2</sub> H <sub>5</sub> Ethyl 3,4-methylenedioxybenzoate	1.0	36	50
R—CH=CH—CO <sub>2</sub> CH(CH <sub>2</sub> ) <sub>5</sub> Cyclohexyl 3,4-methylenedioxybenzoate	1.0	51	36
R—CH=C(CN)CO <sub>2</sub> — <i>o</i> -ClC <sub>6</sub> H <sub>4</sub> <i>o</i> -Chlorophenyl α-cyano-β-3,4-methylenedioxyphenylacrylate	0.5	56	51
R—CH=C(CN)CO <sub>2</sub> — <i>n</i> -C <sub>4</sub> H <sub>9</sub> <i>n</i> -Butyl α-cyano-β-3,4-methylenedioxyphenylacrylate	0.5	53	51
R—CH=CH—CO—C <sub>6</sub> H <sub>5</sub> Piperonalacetophenone	1.0	57	65
R—CH=CH—CO— <i>p</i> -ClC <sub>6</sub> H <sub>4</sub> Piperonal <i>p</i> -chloroacetophenone	1.0	29	56
R—CH <sub>2</sub> —O—CO—NHC <sub>6</sub> H <sub>5</sub> Piperonyl N-phenyl carbamate	1.0	13	43
R—CH <sub>2</sub> —O—CO <sub>2</sub> C <sub>2</sub> H <sub>5</sub> Piperonyl ethylcarbonate	0.5	77	74
$\begin{array}{c} \text{CH—R} \\    \\ \text{CH}_3\text{—CO—C—CO}_2\text{C}_2\text{H}_5 \end{array}$ Piperonalacetoacetic ethyl ester	0.5	52	51
R—CH=C(CO <sub>2</sub> C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub> Diallyl piperonalmalonate	1.0 0.5	65 43	52 50
[CH <sub>3</sub> —CO—CH(CO <sub>2</sub> C <sub>2</sub> H <sub>5</sub> )—] <sub>2</sub> CHR Piperonylidene bis (acetoacetic ethyl ester)	0.5	53	51
R—CH <sub>2</sub> —NHCO(CH=CH) <sub>2</sub> —CH <sub>3</sub> N-Piperonyl sorbamide	2.0	41	49
R—CH <sub>2</sub> —NH—CO—C <sub>4</sub> H <sub>5</sub> O N-Piperonyl furamide	0.5	42	48
$\begin{array}{c} \text{N=C—CH}_3 \\ / \quad \backslash \\ \text{R—CH=C} \quad \text{O} \\ \backslash \quad / \\ \text{O=C—O} \end{array}$ 2-Methyl-4-piperonal-5-oxazolone	0.5	14	25
$\begin{array}{c} \text{R—CH—C—CO}_2\text{C}_2\text{H}_5 \\   \quad    \\ \text{C}_2\text{H}_5\text{O}_2\text{C—CH—N} \\   \quad / \quad \backslash \\ \text{NH} \end{array}$ Diethyl 4-(3,4-methylenedioxyphenyl)pyrazoline-dicarboxylate	1.0	28	50

TABLE I (Continued)

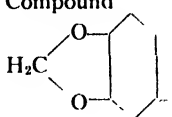
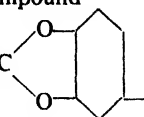
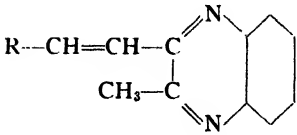
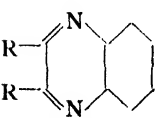
Compound where R is 	Compound, g. per 100 ml.	% Kill (24 hr.)	O.T.I. % kill (24 hr.)
$\begin{array}{c} \text{R}-\text{CH}-\text{CH}-\text{CO}_2\text{C}_2\text{H}_5 \\ \quad \quad \quad   \\ \quad \quad \quad \text{CH} \\ \quad \quad \quad   \\ \quad \quad \quad \text{CO}_2\text{C}_2\text{H}_5 \end{array}$ Diethyl 1-(3,4-methylenedioxyphenyl)cyclopropane-2,3-dicarboxylate	1.0	17	35
$\text{R}-(\text{CH}=\text{CH})_2-\text{CO}_2-\text{C}_6\text{H}_4-\text{C}_6\text{H}_5$ 2-Chloro-4-phenyl phenyl piperate	0.25	18	56
$\text{R}-(\text{CH}=\text{CH})_2-\text{CO}_2-\text{CH}_2-\text{COC}_6\text{H}_5$ Phenacyl piperate	0.1	26	44
$\text{R}-(\text{CH}=\text{CH})_2-\text{CO}_2-\text{C}_6\text{H}_3(\text{Br})_2$ 2,4-Dibromo- $\alpha$ -naphthyl piperate	0.1	25	44
$\text{R}-(\text{CH}=\text{CH})_2-\text{CO}_2-\text{C}_6\text{H}_2(\text{Cl})_4$ 2,3,4,6-Tetrachlorophenyl piperate	0.25	25	56
$[\text{R}-(\text{CH}=\text{CH})_2-\text{CO}]_2\text{O}$ Piperic anhydride	0.5	29	51
$\text{R}-\text{CH}=\text{CH}-\text{CO}-\text{C}(\text{CH}_3)_3$ Piperonalpinacolone	1.0 0.5	60 46	50 52
$\text{R}-\text{CH}=\text{CH}-\text{CO}-\text{CH}(\text{CH}_2)_2$ Piperonal cyclopropyl methyl ketone	1.0 0.5	75 47	37 52
$\text{R}-\text{CH}=\text{CH}-\text{C}_5\text{H}_4\text{N}-\text{CH}=\text{CH}-\text{R}$ 2,6-Bis-(3,4-methylenedioxyphenyl) pyridine	0.5	44	37
$\text{R}-\text{CH}=\text{CH}-\text{CO}-\text{C}(\text{CH}_3)=\text{N}-\text{O}-\text{CH}_2-\text{C}_6\text{H}_5$ 1-(3,4-Methylenedioxyphenyl)-3,4-dione-1-pentene, 4-oxime-O-benzyl ether	1.0	23	35

TABLE I (Continued)

Compound where R is 	Compound, g. per 100 ml.	% Kill (24 hr.)	O.T.I. % kill (24 hr.)
 2-(3,4-Methylenedioxyphenyl)-3-methyl quinoxaline	2.0	41	45
Diisosafole*	1.0	28	56
Protopine alkaloid	0.5	3	26
Podophyllin (N.F.)	0.25	16	46
Apiole (technical)	2.0	22	39
R-CH(OH)-CO-R Piperoin	1.0†	53	53
R-CO-CO-R Piperil	1.0†	39	53
 2,3-Bis-(3,4-methylenedioxyphenyl) quinoxaline	1.0†	34	53
(R-CH=CH-) <sub>2</sub> CO Dipiperonalacetone	1.0†	33	53

\* The structure of the diisosafole (m.p. 145° C.) appears to be controversial.

† Due to the low solubilities of these compounds in most organic solvents, ethyl oxalate was used instead of the acetone-"Deo-base" mixture.

tion in order to ensure the high knockdown necessary in the Peet-Grady procedure. This concentration of pyrethrins, when used alone, was found to give a 19 per cent kill with an O.T.I. kill of 48 per cent.

The spray solutions were tested against houseflies (*Musca domestica* L.) by the standard large group Peet-Grady procedure (4). The kills were compared with those obtained by using the regular Official Test Insecticide (O.T.I.) (3), which contains 0.1 g. pyrethrins per 100 ml., on the same batch of flies. These results are shown in Table I. The 10-minute knockdown values are omitted from the table as in all cases these were of the order of 90 per cent—a result of the addition of pyrethrins to the test solutions.

## SUMMARY

A report is made of the toxicities toward houseflies (*Musca domestica* L.) of sixty-nine organic compounds of various types, all containing the methylenedioxyphenyl group in their structures, when tested in solution with a low concentration of added pyrethrins. The report covers those compounds containing this group which have been found to be relatively inactive or only moderately active. The more active compounds containing this group are reported elsewhere.

The presence of a methylenedioxyphenyl group in the structure of a compound is not a sufficient condition for insecticidal activity or for synergistic activity with pyrethrum.

## ACKNOWLEDGMENT

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# RESPONSE OF LETTUCE SEEDS TO THIOUREA TREATMENTS AS AFFECTED BY VARIETY AND AGE

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## INTRODUCTION

It has been known for some time that the germination of lettuce seeds is affected by germination medium, temperature, moisture, light, carbon dioxide and oxygen relationship, and the variety and age of the seeds. Also it has been noted that nutrition and physiologic age of the lettuce plant influences the germination behavior of the seeds produced. Some of these factors have been described by Thompson (2, 3, 4). Many other workers have contributed to our knowledge in this field but no attempt will be made to review all of their reports here. Lettuce seeds, especially when freshly harvested, require relatively low temperatures for germination, that is, temperatures of 20° C. or below. However, by pretreatment in a moist medium at a low temperature, lettuce seeds can be made to germinate at temperatures well above 20° C. Recent published reports state that germination of lettuce seed at high temperatures can also be stimulated by treatment with thiourea. This was reported first by Thompson and Kosar in 1938 (7). They tried several different chemicals but found that 0.5 per cent thiourea gave the highest germination of Grand Rapids and Hubbard Market varieties. In 1939 and 1944 more extensive reports with essentially the same findings (5, 6, 8) involved the use of fourteen different seed stocks, the varieties of which were not described. Again in 1944 Thompson and Horn (6) obtained marked increases in germination of lettuce seed treated with thiourea and germinated at high temperatures. They treated ten commercial varieties and ten unnamed breeding stocks and found that percentage solution used, soaking temperature, and duration of soaking all affected the results. They obtained highest germination at 33° to 35° C. after pre-soaking seeds for seven hours in darkness in a 0.5 per cent solution of thiourea at 18° C. Seeds were then washed in tap water and dried ten days to two weeks before germination. No germination tests were made immediately after the thiourea treatment. In spite of differences in results obtained from different varieties, thiourea treatment gave consistently higher germination percentages at high temperatures than water-soaked controls.

Raleigh (1), repeating these thiourea treatments in light and in darkness, showed that freshly harvested seeds of seven strains of *Lactuca serriola* L. required light in addition to thiourea treatment for stimulation of germination at high temperatures. Thiourea treatments in the dark as well as in the light were effective for three strains of *Lactuca sativa* L.

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In the reports of Thompson and Kosar (7, 8), Thompson (5), and Thompson and Horn (6), no mention was made of the age of the lettuce seed used for the tests. Preliminary tests made on freshly harvested lettuce (*Lactuca sativa* L.) seeds at Boyce Thompson Institute gave indications that age of the seeds was an important factor in the effectiveness of thiourea in stimulating germination at high temperatures. Results of these preliminary trials and more extensive experiments to test this finding are presented here.

## PROCEDURE AND RESULTS

### 1944 CROP

Lettuce seeds of the variety Iceberg were harvested August 1, 1944 to August 18, 1944, from plants grown at Boyce Thompson Institute. Seeds harvested throughout the period were mixed and spread out on blotting paper in the laboratory until thoroughly dry. They were then cleaned and stored at room temperature in a manila envelope.

On September 17, 1944 seeds were soaked for seven hours at 20° C. in darkness in a 0.5 per cent aqueous solution of thiourea and in tap water. Lots of 100 were germinated immediately after treatment on moist filter paper at constant temperatures of 20°, 30°, and 35° C. One hundred per cent of the seeds at 20° C. germinated, but no seeds at 30° or 35° C. germinated. Similar tests were then made about every four weeks on seeds stored dry in the laboratory after harvest.

Lots of 100 seeds were placed in Petri dishes on filter paper moistened with tap water, and 0.5 and 1.0 per cent solutions of thiourea respectively, and germinated in darkness at constant temperatures of 20° and 30° C. At the same time seeds were put to soak in 15 cm. Petri dishes in tap water and in 0.5 and 1.0 per cent solutions of thiourea. These seeds were soaked for seven and sixteen hours in darkness at a constant temperature of 20° C. Following soaking, seeds were removed from solution and placed, without washing, in 9.5 cm. Petri dishes on filter paper moistened with tap water. As before, seeds were germinated at 20° and 30° C.

Seeds soaked in 0.5 and 1.0 per cent thiourea and germinated at 30° C. showed a progressive increase in germination with increased periods of dry storage after harvest. Seeds soaked sixteen hours in 1.0 per cent thiourea gave 20 per cent germination after 26 days and 97 per cent germination after 235 days dry storage. Soaking in 0.5 per cent thiourea for sixteen hours resulted in 2 per cent germination after 26 days storage as compared with 100 per cent after 235 days storage. At 20° C., all lots gave between 90 and 100 per cent germination, regardless of the length of the storage period. Similar increases with dry storage were obtained by germinating seeds on filter paper moistened with thiourea but only with considerable injury to the seedlings.

These tests indicated that the effectiveness of the thiourea treatment depended on the age of the seeds used.

#### 1945 CROP

On the basis of the results of tests on the 1944 crop of lettuce seeds, a second more extensive series of tests was set up. Seeds of the varieties White Boston, Grand Rapids, Iceberg, and Black-seeded Simpson, sent from W. Atlee Burpee Company, Ventura, California, immediately after harvest were used. This meant that six to ten days elapsed between the date of harvest and the first test. Upon receipt, seeds were well mixed and divided into six equal lots. One lot was used immediately. The other five lots were stored dry in open bottles at room temperature until tested. Grand Rapids seeds were tested after 0, 1, 2, 4, 8, and 16 weeks storage. The other three varieties were tested after 0, 1, 4, 8, and 16 weeks storage.

For each test, estimated lots of 800 seeds were soaked in 15 cm. Petri dishes at 20° C. and 30° C., in tap water, 0.5 and 1.0 per cent solutions of thiourea, for three, seven, and sixteen hours. Following the soaking period, seeds were drained and rinsed in tap water. Duplicate lots of 100 seeds were counted and germinated on filter paper moistened with tap water in 9.5 cm. Petri dishes in darkness at constant temperatures of 20° and 30° C. The remaining treated seeds were spread on filter paper and dried quickly. These were stored in the laboratory in Petri dish halves for 10 or 11 days after which time duplicate lots of 100 were germinated at 20° and 30° C.

In like manner, estimated lots of 400 seeds were soaked in tap water at 5° C. for 24 and 48 hours. Duplicate lots of 100 of these were germinated at 30° C. immediately, and after 10 or 11 days dry storage. Counts were made every day or every other day for a week.

Concurrently with soaking treatments, duplicate untreated lots of 100 seeds were germinated in darkness in Petri dishes on moist filter paper at constant temperatures of 10°, 15°, 20°, 25°, and 30° C., and at alternating temperatures of 10° to 20° C., 10° to 30° C., 15° to 30° C., and 20° to 30° C. In the case of each alternating temperature, the seeds were placed at the lower temperature first and thereafter left at the low temperature for sixteen hours and at the high temperature for eight hours daily. Germination tests at 10°, 15°, 10° to 20°, and 10° to 30° C. were eliminated after the first test because germination percentages at these temperatures were above 90. Results of germination tests for the other temperatures are presented in Table I. All germination percentages given in this paper represent averages obtained from duplicate cultures of 100 seeds each. It is evident from the data in Table I that seeds of these four varieties of lettuce are never dormant in the sense that they will not germinate if suitable temperature is supplied. White Boston was the most dormant variety, as measured by its failure to germinate at both 25° and 30° C., and reduced germination

even at 20° C. up to four weeks of storage. Iceberg, on the other hand, germinated well at all temperatures but 30° C., and after sixteen weeks storage gave 38 per cent germination at that temperature. Grand Rapids and Black-seeded Simpson varieties were somewhat intermediate in their germination requirements. Both of these gave some germination at 25° C. even when fresh. It would be expected then that Iceberg lettuce would respond almost immediately to thiourea treatment, whereas the other three varieties would respond only after increased periods of dry storage. There

TABLE I  
GERMINATION OF FOUR VARIETIES OF UNTREATED LETTUCE SEEDS AT  
VARIOUS TEMPERATURES

Variety	Germ. temp., ° C.	Per cent germination after weeks of dry storage				
		0	1	4	8	16
White Boston	20	69	33	100	99	100
	25	2	1	1	1	5
	30	0	0	0	0	0
	15 to 30*	97	42	98	95	100
	20 to 30*	4	2	2	58	22
Grand Rapids	20	92	97	96	94	95
	25	11	25	28	47	87
	30	0	2	0	1	3
	15 to 30*	98	97	94	97	96
	20 to 30*	91	92	92	94	96
Iceberg	20	96	97	95	97	97
	25	90	95	90	95	94
	30	1	0	11	6	38
	15 to 30*	98	97	97	97	97
	20 to 30*	99	97	99	99	97
Black-seeded Simpson	20	87	94	98	98	96
	25	49	5	9	51	41
	30	0	1	1	1	0
	15 to 30*	90	79	99	96	95
	20 to 30*	90	75	91	89	94

\* Daily alternation. Cultures left at the lower temperature for sixteen hours and at the higher temperature eight hours each day.

was no variation in experimental procedure to account for the comparatively low germination of seeds of White Boston lettuce at 20° C. and 15° to 30° C., after one week of dry storage or for some apparent inconsistencies in the germination behavior of Black-seeded Simpson seeds.

Germination tests were carried out at constant temperatures of both 20° and 30° C. The purpose of germinating treated seeds at 20° C., a favorable germination temperature, was to determine possible injurious effects of the chemical treatment. No such effects were observed in spite of an early indication that some injury might follow drying of thiourea-treated, non-dormant seeds. The germination percentages obtained at 20° C. were

above 90 per cent for all varieties and treatments and hence are not included in this paper.

Results of presoaking four varieties of lettuce seeds on their subsequent germination at 30° C. are shown in Tables II, III, IV, and V. The figures represent germination of seeds removed from the presoaking solution,

TABLE II  
WHITE BOSTON 1945. GERMINATION OF SEEDS PLACED AT 30° C. IMMEDIATELY  
AFTER TREATMENT

Soaking			Per cent germination after weeks of dry storage				
Medium	Temperature, ° C.	Time, hours	0	1	4	8	16
Tap water	20	7	0	0	1	3	1
		16	3	0	8	24	48
	30	7	0	0	0	0	0
		16	0	0	0	0	0
Thiourea 0.5%	20	7	1	0	1	4	20
		16	16	7	43	87	96
	30	7	0	0	0	0	0
		16	0	0	0	1	4
Thiourea 1.0%	20	7	0	0	5	9	26
		16	16	12	49	86	97
	30	7	1	0	1	1	10
		16	8	8	8	30	43
Tap water	5	24	6	0	4	23	22
		48	68	26	90	86	78

washed, and then placed immediately to germinate on filter paper moistened with tap water. Although a three-hour soaking period was used in each case, the resulting data were omitted from the tables since they approximated those obtained after seven hours of soaking.

It is obvious from a glance at the data shown in these tables that soaking in thiourea, either 0.5 or 1.0 per cent solution, is superior to soaking in tap water at both 20° and 30° C. for stimulating germination at the unfavorable temperature of 30° C.

The increasing effectiveness of presoaking with the lengthening of the period in dry storage and hence the increase in age of the seed before treatment is also apparent. This is one of the most striking effects noted in the present experiments, and applies to both tap water and thiourea soaking. Seeds of the White Boston variety, for example, treated as soon as received in this laboratory gave 3 and 16 per cent germination at 30° C. when presoaked at 20° C. for sixteen hours in tap water and 0.5 per cent

thiourea respectively (Table II). Soaking in tap water at 5° C. for 48 hours was superior to either of these treatments. Seeds from the same original lot stored dry in the laboratory for eight weeks before receiving the same treatment gave 24 and 87 per cent germination. At this time the thiourea treatment was equal to soaking in tap water for 48 hours in its stimulatory effect. Similar results are to be seen for the other varieties, even for Iceberg, the least dormant (Tables III, IV, V). Thus it is seen that any con-

TABLE III  
GRAND RAPIDS 1945. GERMINATION OF SEEDS PLACED AT 30° C. IMMEDIATELY  
AFTER TREATMENT

Soaking			Per cent germination after weeks of dry storage					
Medium	Temperature, ° C.	Time, hours	0	1	2	4	8	16
Tap water	20	7	2	1	8	2	2	14
		16	3	3	5	6	11	31
	30	7	1	1	1	0	3	4
		16	0	0	1	0	0	2
Thiourea 0.5%	20	7	5	17	12	3	11	43
		16	17	24	22	30	36	56
	30	7	2	2	2	0	3	7
		16	2	0	0	1	7	2
Thiourea 1.0%	20	7	6	16	11	8	8	37
		16	15	21	19	32	30	40
	30	7	4	1	2	2	3	11
		16	3	6	4	1	9	17
Tap water	5	24	26	46	12	11	40	40
		48	17	11	26	28	58	63

clusion as to stimulation of germination of lettuce seeds by presoaking in solutions of thiourea would depend not only upon the variety used but the length of time which had elapsed between harvest and testing date.

Other factors are also important of course. Comparing the effects of soaking for seven and sixteen hours it will be noted that the latter was to be preferred in many cases. Exceptions to this are to be seen in soaking of seeds of White Boston, Grand Rapids, and Black-seeded Simpson varieties at 30° C. in both tap water and 0.5 per cent thiourea where practically no stimulation resulted and in the soaking of seeds of the less dormant Iceberg variety.

Also soaking at 20° C. was much more effective than soaking at 30° C. Considering White Boston variety (Table II), seeds failed to germinate at 30° C. after soaking in tap water or 0.5 per cent thiourea at 30° C. for

seven or sixteen hours. When the concentration of the thiourea solution was increased to 1.0 per cent, stimulation of germination up to 43 per cent for seeds stored sixteen weeks before treatment was obtained by soaking

TABLE IV

ICEBERG 1945. GERMINATION OF SEEDS PLACED AT 30° C. IMMEDIATELY AFTER TREATMENT

Soaking			Per cent germination after weeks of dry storage				
Medium	Temperature, ° C.	Time, hours	0	1	4	8	16
Tap water	20	7 16	59 31	28 73*	41 84*	79 88*	93 94*
	30	7 16	1 0	2 1	1 0	17 4	36 26
Thiourea 0.5%	20	7 16	84 80	68 88	74 92	86 96	95 95*
	30	7 16	15 6	7 10	24 48*	55 45	78 64
Thiourea 1.0%	20	7 16	80 76	70 82	73 92	87 98	94 95
	30	7 16	14 15	7 18	40 74	55 74	84 78
Tap water	5	24 48	88 90	88 93	93 93	87 88	85 94

\* Seeds germinating at the end of the soaking period.

at 30° C. Similar effects were observed for Grand Rapids and Black-seeded Simpson varieties (Tables III and V).

Some of the effects obtained are shown graphically in Figures 1 to 4. Stimulation by thiourea treatment was obtained from seeds soaked at 20° C. for sixteen hours in 0.5 per cent thiourea. Figure 1 is a comparison of germination percentages of the four varieties given this treatment. Treated Iceberg seeds gave consistently high germination and even shortly after harvest gave 80 per cent germination. Treated White Boston, however, germinated poorly at 30° C. when first received. After four weeks dry storage 43 per cent of the seeds germinated after thiourea treatment. With the extension of the dry storage of White Boston seeds to eight weeks before soaking in thiourea, germination at 30° C. was increased to 87 per cent. Both Grand Rapids and Black-seeded Simpson varieties showed more gradual germination increases with increased dry storage, attaining 56 and 63 per cent respectively after sixteen weeks of storage.

Figures 2 and 3 are graphs which compare the results obtained by soak-

TABLE V  
BLACK-SEEDED SIMPSON 1945. GERMINATION OF SEEDS PLACED AT 30° C.  
IMMEDIATELY AFTER TREATMENT

Soaking			Per cent germination after weeks of dry storage				
Medium	Temperature, ° C.	Time, hours	0	1	4	8	16
Tap water	20	7 16	0 10	1 6	0 21	3 23	9 24
	30	7 16	0 1	1 1	1 0	0 0	1 0
Thiourea 0.5%	20	7 16	1 29	0 22	4 52	17 57	27 63
	30	7 16	0 1	0 1	1 2	0 1	11 4
Thiourea 1.0%	20	7 16	4 33	1 21	6 46	38 62	42 63
	30	7 16	2 7	3 7	9 7	3 15	19 38
Tap water	5	24 48	9 52	18 58	42 —	58 79	22 70

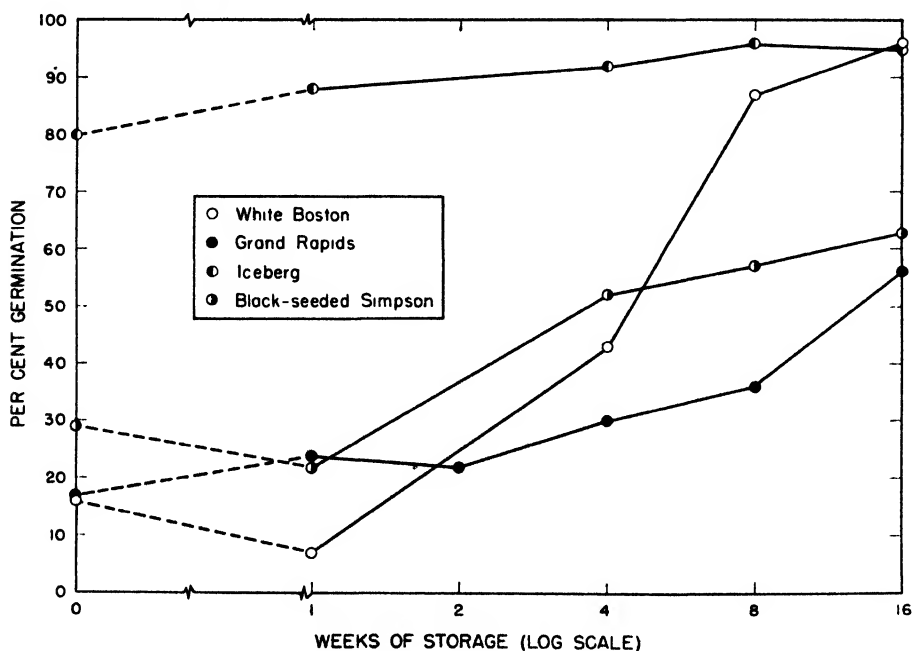


FIGURE 1. Germination of four varieties of lettuce seeds soaked for 16 hours at 20° C. in 0.5 per cent thiourea and placed immediately at 30° C.

ing Iceberg and Black-seeded Simpson at 20° and 30° C. for sixteen hours in 1.0 per cent thiourea with those obtained by soaking in tap water. Soaking at 20° C. in tap water resulted in a corresponding increase in germination at 30° C. although stimulation by water soaking was less than

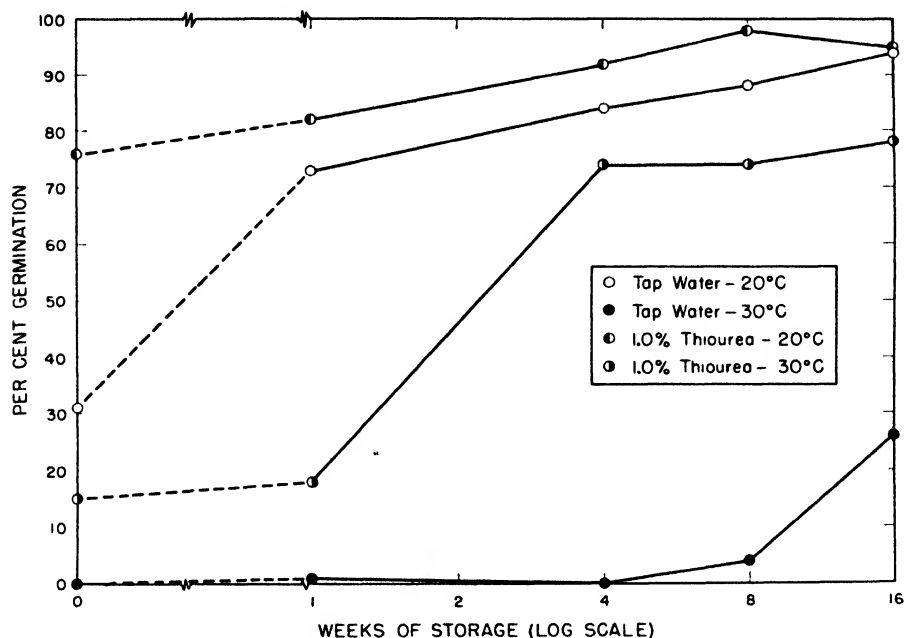


FIGURE 2. Germination of Iceberg lettuce seeds soaked for 16 hours at 20° C. and 30° C. in tap water and in 1.0 per cent thiourea and placed at 30° C. immediately.

by thiourea soaking. As with thiourea treatment, however, germination increased as period of dry storage was lengthened.

Seeds germinated at 30° C. following a drying period of 10 or 11 days failed to give as good germination as those germinated immediately after treatment. Table VI gives a comparison, for the four varieties used, of the effect of drying on the germination of seeds soaked sixteen hours at 20° C. in tap water and in 0.5 and 1.0 per cent solutions of thiourea.

Treated seeds which were dried 10 or 11 days before germination at 30° C. showed a decrease in germination as compared with seeds germinated immediately. Again the expected differences among varieties and the age of the seeds is seen. Although the germination of fresh seeds is stimulated by thiourea presoaking, this effect does not hold over if the treated seeds are dried 10 or 11 days in the laboratory before germinating. For example, fresh seeds of Grand Rapids gave 17 and 15 per cent germination following presoaking in thiourea solutions when they were tested for germination immediately following treatment. Drying the pretreated seeds for



TABLE VI

GERMINATION OF FOUR VARIETIES OF LETTUCE SEEDS SOAKED AT 20° C. FOR SIXTEEN HOURS AND GERMINATED AT 30° C. IMMEDIATELY AND AFTER DRY STORAGE

Variety	Soaking medium	Per cent germination after weeks of dry storage									
		0		1		4		8		16	
		A*	B**	A*	B**	A*	B**	A*	B**	A*	B**
White Boston	Tap water	3	2	0	0	8	11	24	23	48	18
	Thiourea, 0.5%	16	4	7	3	43	41	87	64	96	80
	" 1.0%	16	4	12	9	49	72	86	85	97	94
Grand Rapids	Tap water	3	1	3	1	6	1	11	1	31	13
	Thiourea, 0.5%	17	1	24	4	30	16	36	25	56	28
	" 1.0%	15	1	21	13	32	12	30	16	40	24
Iceberg	Tap water	31	10	73†	32†	84†	75†	88†	76†	94†	72†
	Thiourea, 0.5%	80	18	88	49	92	90	96	92	95†	88†
	" 1.0%	76	29	82	60	92	94	98	92	95	93†
Black-seeded Simpson	Tap water	10	2	6	1	21	3	23	7	24	10
	Thiourea, 0.5%	29	2	22	1	52	5	57	16	63	6
	" 1.0%	33	1	21	3	46	8	62	38	63	22

\* Germinated immediately.

\*\* Dried ten or eleven days before germination.

† Seeds germinating at the end of the soaking period.

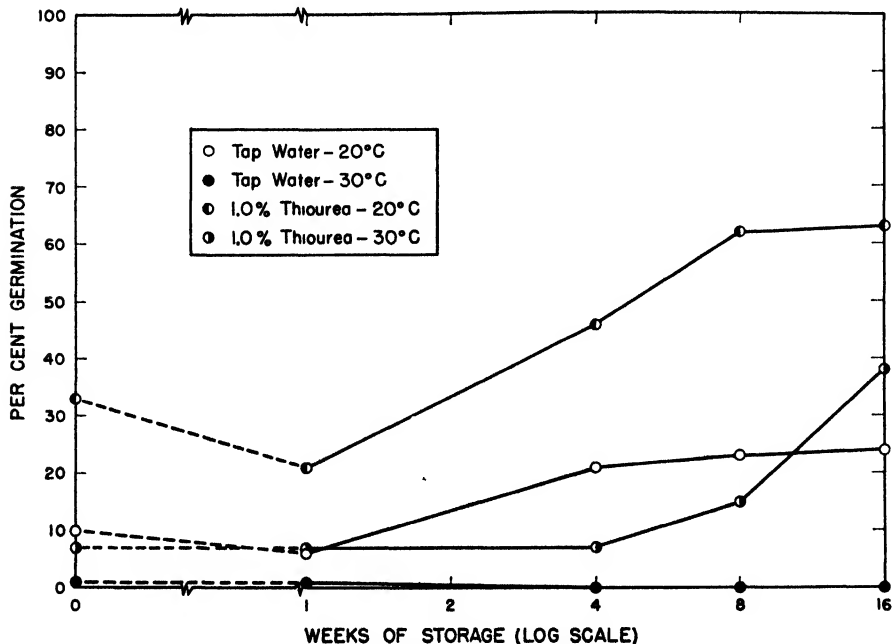


FIGURE 3. Germination of Black-seeded Simpson lettuce seeds soaked for 16 hours at 20° and 30° C. in tap water and in 1.0 per cent thiourea and placed at 30° C. immediately.

10 days before germination eliminated the stimulatory action of the chemical, only 1 per cent of the seeds germinating under these conditions. Graphic presentation of some of these data is shown in Figure 4. At first treated Iceberg seed which had been dried gave very low germination, but with increased time after harvest the differences caused by drying were very small. Consistently low germination percentages were obtained, on

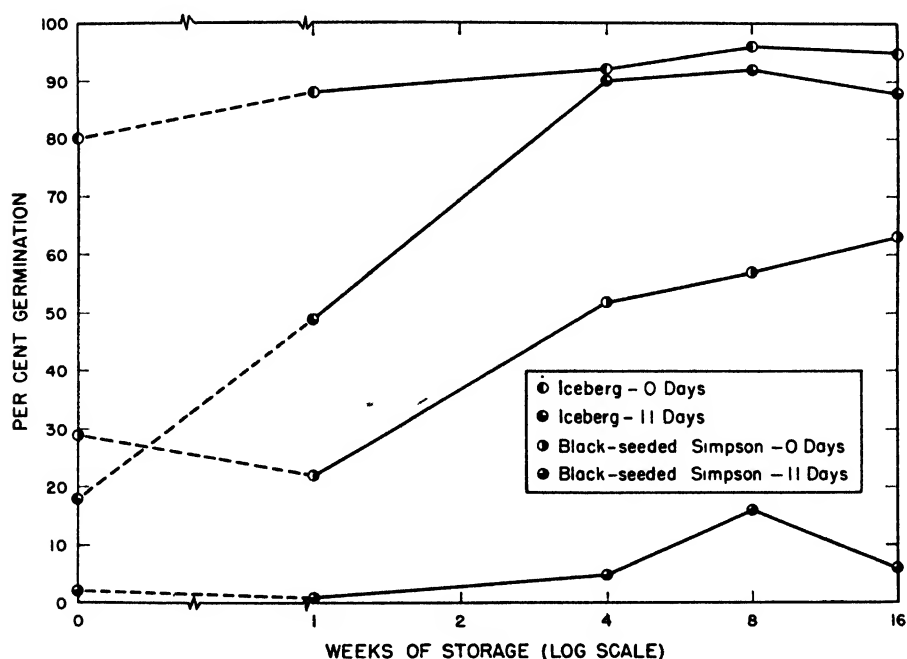


FIGURE 4. Germination of Iceberg and Black-seeded Simpson lettuce seeds soaked 16 hours at 20° C. in 0.5 per cent thiourea and placed at 30° C. immediately, and after a drying period.

the other hand, from Black-seeded Simpson seed which had been dried following thiourea treatment. As can be seen from the graph increased periods of dry storage of these seeds had little effect in increasing germination of treated seeds which had been dried.

In an attempt to account for poor germination of treated seeds which had been dried, some treated seeds were germinated in Petri dishes in a layer of water. However, this method gave no better results.

These results differ in several respects from those reported by Thompson and his co-workers. For greatest stimulation without injury they used 0.5 per cent thiourea with a soaking period of seven hours, whereas a sixteen-hour soaking period proved most effective in the present tests. When seeds were soaked at 30° C. in the present tests a 1.0 per cent

thiourea solution stimulated germination to a greater extent than a 0.5 per cent solution. Thompson and Horn on the other hand found 0.5 per cent solution to be preferable for soaking at temperatures up to 27° C. Thompson and Horn (6) using Black-seeded Simpson seeds in one test found that thiourea soaking stimulated germination at 33° to 35° C. even after a drying period of three weeks following treatment. Black-seeded Simpson used in the present experiment germinated fairly well at 30° C. immediately after treatment but poorly after a drying period of 10 to 11 days. Of the other three varieties, only Iceberg seeds gave high germination at 30° C. when dried 10 or 11 days following thiourea treatment. The stimulatory effect of thiourea treatment on White Boston and Grand Rapids seeds failed to carry over for a drying period of 10 or 11 days. Here again the age of the seed may be a factor in the holding over of the thiourea stimulation for dormant seeds or those which have specific temperature requirements for germination at the time of harvest. Differences in the age of seed from harvest and varietal differences may account in part for the differences in results obtained by Thompson and his co-workers and those reported here. Variations in procedure may also have had an effect. This might be true especially of the amount of washing given the treated seeds before drying them.

#### SUMMARY

Four varieties of lettuce seeds (White Boston, Grand Rapids, Iceberg, and Black-seeded Simpson) which were soaked in 0.5 and 1.0 per cent thiourea solutions and germinated immediately after treatment displayed a gradual increase in germination with the lengthening of the period of dry storage between harvest and treatment. Differences in variety and age of the seeds caused variation in amount of stimulation by thiourea treatment. Germination at 30° C. of freshly harvested Iceberg seeds which had been treated, was considerably higher than germination of the other three more dormant varieties.

Presoaking seeds in a 0.5 or 1.0 per cent thiourea solution for sixteen hours at 20° C. resulted in greatest stimulation of germination at 30° C. Soaking at 20° C. was more effective than soaking at 30° C. when seeds were germinated at 30° C. Seeds soaked at 30° C. germinated best when treated with 1.0 per cent thiourea solution.

Seeds of the four varieties which were dried 10 or 11 days following thiourea treatment gave lower germination at 30° C. than seeds which were germinated immediately. The amount of this decrease depended on the variety and age of the seeds.

Seeds soaked in tap water at 5° C. for 48 hours and placed at 30° C. immediately germinated almost as well as thiourea-treated seeds.

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# EFFECT OF AGE AND STORAGE CONDITION OF SEEDS ON THE YIELDS OF CERTAIN PLANTS

LELA V. BARTON AND HELEN R. GARMAN

The increasing practice among seedsmen of keeping surplus supplies of seeds for sale in subsequent years has resulted from the experimental determinations of the storage conditions effective for the maintenance of viability of the several types of seeds. Since numerous reports dealing with specific effects on seed quality of temperature, moisture, and gaseous exchange have appeared in the literature, no attempt will be made to review them here. For the most part the measure of the keeping quality has been the power of the seeds to produce seedlings as determined by germination percentages obtained from soil or other plantings. Since the establishment of favorable storage conditions by numerous workers for many kinds of seeds, more attention is being placed on the kind of plants which may be expected to develop from seeds of different ages and storage conditions.

The present investigation was undertaken to determine whether the age and storage condition of the seeds of aster (*Callistephus chinensis* Nees.), verbena (*Verbena teucrioides* Gill. & Hook.), pepper (*Capsicum frutescens* L. [*C. annuum* L.]), tomato (*Lycopersicon esculentum* Mill.), and lettuce (*Lactuca sativa* L.) would have an effect on the yield of plants grown from such seeds.

## MATERIAL AND METHODS

In order to have fresh seeds of the same genetic strain to compare with those which had been stored for longer periods, plants of aster, verbena, pepper, tomato, and lettuce were grown in 1944 from seeds of the original lots which had been stored several years before. From these plants seeds were collected, cleaned, dried, and stored in the laboratory until April, 1945. All of the fresh seeds, except for lettuce, were produced in the greenhouse and pollination was controlled. Lettuce seeds were produced in the field but no other lettuce flowered in the vicinity; hence the strain remained pure. Very fresh seeds of pepper and tomato were secured for planting in April, 1945 from plants (also produced from old seeds of the original lot) grown in the greenhouse during the winter of 1944-1945.

Aster and verbena seeds were received from W. Atlee Burpee Company in October, 1935. The effect of various storage conditions on the germination capacity of these seeds has been reported (4). Those seeds of aster and verbena with approximately 4 per cent moisture content stored in tin cans at  $-5^{\circ}$  C. were used in the present tests. Plants of both of these forms were grown in the field in 1945 from seeds as follows: lot A, seeds produced in

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the greenhouse during the summer of 1944 and lot B, seeds from the original stored lots. Thus the seeds used to produce plants in lot A were approximately seven months old while those used for lot B were approximately nine and one-half years old.

Pepper, tomato, and lettuce seeds were obtained from the Kilgore Seed Company in May, 1932 at which time each lot was divided and stored under various conditions. Two published articles deal with storage effects on the germination capacity of these seeds (2, 3).

Pepper seeds which had been mixed with calcium oxide to remove one-half of the original moisture and then sealed in tin cans and stored at  $-5^{\circ}$  C. were selected for these tests. To produce plants for field performance tests in 1945, fresh and old seeds were used as follows: lot A, seeds produced in the greenhouse in the spring of 1945 from plants grown from the seeds stored in 1932, as described above. These seeds were harvested not more than two weeks before sowing for the field test; lot B, seeds produced in the greenhouse in the summer of 1944 from plants grown from the seeds stored in 1932 as described above. These seeds were about eight months old when they were planted for the field trials; lot C, seeds from the original lot described above stored in 1932. Thus it is seen that pepper plants grown in the field in 1945 represented those from very fresh seeds (lot A), those from 8-month-old seeds (lot B), and those from 13-year-old seeds (lot C), all from the identical genetic strain.

Tomato seeds were similar to those described for pepper except that two original storage conditions were used instead of one. This was to permit comparison of field performance of plants from old seeds stored under favorable and unfavorable conditions as well as from fresh and old seeds. Seeds of lot C had been stored for thirteen years in open containers in the laboratory. Lots A and B were produced from C in the same manner as described for pepper. Seeds of lot F had been mixed with calcium oxide to remove one-half of the moisture and then sealed in tin cans and stored at  $-5^{\circ}$  C. for thirteen years. Lots D and E were produced from F in the same manner as A and B were produced from C.

In the case of lettuce, as for tomato, seeds from two original storage conditions served as bases. Both lots had been mixed with calcium oxide to remove one-half of the moisture but lot B was stored in the laboratory while lot D was stored at  $-5^{\circ}$  C. Lots A and C were produced in the summer of 1944 from plants which had been grown from lots B and D respectively.

Seeds of all varieties were planted in soil in flats and placed in the greenhouse in April, 1945. As germination occurred and the seedlings grew, they were transplanted to individual pots from which they were set in the field.

The flowers produced by aster and verbena plants were taken as a measure of the effect of age and storage conditions of the seeds from which

they were produced. The weights of fruits produced by pepper and tomato plants were recorded and the lettuce heads were weighed at the time when a majority of them had reached the best stage for marketing.

## RESULTS

### ASTER

Plants from seed lots A (seven months old) and B (nine and one-half years old) were set in the field on May 29, 1945. The planting consisted of eight rows with 22 plants each arranged as follows:

ABBAABB—————B  
BAABBAA—————A  
etc.

The flowers appearing on the plants were cut one to three times per week and a record kept of the number from each plant. The first flowers appeared on July 18. The experiment was terminated on August 27 at which time most of the plants were dead or dying. Plants from lot A produced 3,461 and those from lot B, 3,378 flowers during the season (Table I). These

TABLE I  
CUMULATIVE WEEKLY TOTALS OF ASTER FLOWERS CUT DURING THE GROWING  
SEASON OF 1945

Row	Lot A from seeds 7 months old							Lot B from seeds 9.5 years old						
	Weeks							Weeks						
	1	2	3	4	5	6	7	1	2	3	4	5	6	7
1	1	23	148	331	475	533	546	8	84	239	337	382	432	441
2	0	22	105	209	319	366	384	2	30	114	199	261	295	311
3	1	22	123	252	371	442	473	14	66	205	344	421	451	461
4	1	27	139	212	346	426	432	5	58	104	344	456	525	543
5	3	37	148	240	330	378	384	6	65	205	335	398	436	449
6	2	20	176	323	428	483	498	4	58	173	264	352	414	424
7	0	15	128	239	324	402	417	5	53	162	210	236	262	268
8	0	8	91	173	267	314	327	3	55	205	365	441	475	481
Totals	8	183	1058	1979	2860	3344	3461	47	469	1497	2398	2947	3290	3378

totals indicate no differences in productivity of plants from fresh or old seeds. However, if earliness in flowering is considered, the plants from the old seeds (lot B) were ahead of those from fresh seeds (lot A) for the first two or possibly three weeks. This shows, not only in the totals, but in the number of flowers cut from each row, though the yield from the rows varied in lot A from 327 to 546 flowers and in lot B from 268 to 543 flowers for the season. There was considerable variation in the flowering of the plants in different parts of the field, but lots A and B were equally affected by good or poor growth conditions.



## VERBENA

Because of the growth habits of these plants and the difficulty of securing exact data on their productivity, 88 plants each of lots A and B were grown side by side. No observable differences in the two lots appeared during the growing season of 1945.

## PEPPER

Plants from the three lots of seeds described under "Material and Methods" were set in the field on May 29, 1945. For the arrangement of plants in the field a standard 3×3 form for a Latin square was used with reshuffling of rows, columns, and treatments to form ten blocks of nine plants each, three plants from each age of seed. The yield measured in weight of pepper fruits produced in each of the ten blocks is shown in Table II. All fruits except the final harvest were removed from the plants

TABLE II  
TOTAL PEPPER FRUIT YIELDS IN OUNCES FOR SEED LOTS AND BLOCKS FOR THE  
ENTIRE SEASON

Lot	Age of seeds	Block										Totals
		1	2	3	4	5	6	7	8	9	10	
A	Fresh	63	63	112	105	73	40	118	56	125	74	829
B	8 mos.	81	79	52	53	63	59	95	77	85	109	753
C	13 yrs.	34	93	60	108	59	39	91	34	87	125	730
Totals		178	235	224	266	195	138	304	167	297	308	2312

and weighed when they were just beginning to turn red. Lots A, B, and C were produced from fresh, 8-month-old, and 13-year-old seeds, respectively.

It is immediately apparent that the block or position in the field influenced the yield. A total of only 138 ounces was produced in block 6, for example, while block 10 produced 308 ounces of fruit. Differences in yield of plants from the three seed lots appeared very small and of doubtful significance. An analysis of variance test of these data confirmed the above findings.

The total amounts of fruits remaining on the plants were harvested on September 21, 1945. These were graded for size and photographed immediately after harvest. It will be seen from Figure 1 that more large fruits were harvested from lot A, or the plants from fresh seeds, on this date. Weights of these fruits were included in the totals of Table II.

A measurement of the heights of the plants made on July 18 also failed to reveal any differences in the lots. A somewhat greater number of fruits were produced by lot A.

## TOMATO

It will be recalled that six lots of tomato seeds were sown for the measurement of yield as follows: lot A, fresh seeds and lot B, 8-month-old seeds both produced from the original lot C which had been stored for 13 years in open containers at room temperature when the field tests were begun; lot D, fresh seeds and lot E, 8-month-old seeds both produced from the original lot F which had been stored for 13 years sealed with calcium oxide at  $-5^{\circ}\text{C}$ . when the field tests were begun.

The seedlings were set in the field on May 22, 1945 except nine plants of lot C which were planted in the field on June 12. This was necessitated by the failure of the seeds of this lot to give the expected germination percentage and the consequent need for a second planting. These nine plants were those of blocks 2 and 3. It may be seen from their productivity as compared with plants of lot C in the other blocks (Table III) that there

TABLE III  
TOTAL TOMATO FRUIT YIELDS IN OUNCES FOR SEED LOTS AND BLOCKS  
FOR THE ENTIRE SEASON

Lot	Age of seeds	Block						Totals
		1	2	3	4	5	6	
A	Fresh	544	568	798	708	543	658	3,810
B	8 mos.	643	397	734	738	594	599	3,705
C	13 yrs.*	299	203	330	470	260	274	1,845
D	Fresh	592	650	697	662	519	635	3,755
E	8 mos.	645	451	814	599	367	713	3,589
F	13 yrs.†	608	712	646	680	478	795	3,919
Totals		3331	2981	4028	3857	2761	3674	20,632

\* Stored in open containers in the laboratory.

† Stored with reduced moisture content in sealed containers at  $-5^{\circ}\text{C}$ .

was no significant effect of the delay in planting. Seeds of lot C were slow in germination due to their decreased germination capacity by improper storage. Consequently the plants were slower in development than those of the other lots. This may be seen in Figure 2 which illustrates a typical plant of each lot on May 22 when they were transferred from the greenhouse to the field.

Fruits were harvested when ripe and the number of fruits and their weights were taken for each plant. Pickings were made on 24 dates during the growing season. On September 25 when there was danger of frost, all of the green fruits remaining were harvested. The weights are included in the figures for total yield.

An examination of the total yield of ripe fruit from each lot (Table III) shows the decidedly inferior production capacity of lot C. Also certain

parts of the field were better than others for tomato production as indicated by the differences in yield of the blocks. Each block represents a random reshuffling of rows, columns, and treatments of a standard  $6 \times 6$  form for Latin squares. The six replications of blocks were equal to the number of

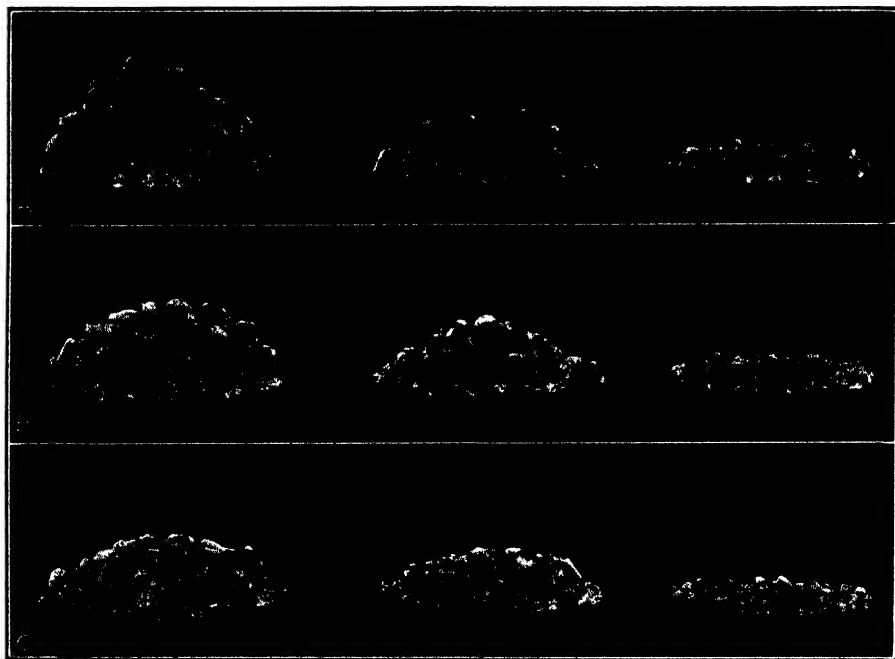


FIGURE 1. Complete harvest of fruits remaining on pepper plants September 22, 1945. Each pile of peppers is 20" at the base. Top to bottom, lots A, B, and C from plants produced from fresh, 8-month-old, and 13-year-old seeds, respectively.

treatments, in this case different seed lots used for plant production, and also to the number of rows. One tomato plant was placed in each cell of the Latin squares.

An analysis of variance was made using the weights of tomato fruits obtained from each plant in the field. The results of this analysis are shown in Table IV. The difference in lots was highly significant as was also the difference between Latin squares. A detailed analysis reveals the source of the differences in lots. The  $F$  value of lots C vs. F shows the advantage of a good storage temperature,  $-5^{\circ}\text{C}.$ , combined with drying and sealing over storage in the laboratory. Both of these seed lots were from the same original source and both had been stored for thirteen years before they were planted. Thus it is demonstrated once again that the actual age of the seed is of much less importance than the environment in which it is held.

The behavior of plants from seeds of lot C is compared with those from lots A and B, both of which had been produced from C but used fresh or stored for only eight months in Table IV, C vs. A+B. A highly significant



FIGURE 2. The appearance of tomato plants at the time they were set in the field on May 22, 1945. Left to right: typical plants produced from seed lots A, B, C, D, E, and F. (Lot A, fresh seeds and lot B, 8-month-old seeds both produced from the original lot C which had been stored for 13 years in open containers at room temperature when the field tests were begun; lot D, fresh seeds and lot E, 8-month-old seeds both produced from the original lot F which had been stored for 13 years sealed with calcium oxide at  $-5^{\circ}\text{C}$ . when the field tests were begun.)

F value was obtained. This shows the decreased vigor of the seeds of lot C when ability to produce high-yielding plants is concerned. But from the fruits which were formed good seeds were obtained (lots A and B). There

TABLE IV  
WEIGHT OF TOMATO FRUITS. ANALYSIS OF VARIANCE

Source of variation	D. F.	Sum of squares	Mean squares	F values
Lots				
C vs. F	1	16,607.5	16,607.5	13.97**
C vs. A+B	1	68,053.5	68,053.5	57.24**
A vs. B	1	1,129.8	1,129.8	0.95
F vs. D+E	1	180.5	180.5	0.15
D vs. E	1	382.7	382.7	0.32
Blocks	5	34,943.9	6,988.8	5.88**
Rows	30	45,643.7	1,521.5	1.28
Columns	30	41,179.3	1,372.6	1.15
Error within blocks	120	133,549.0	1,112.9	0.94
Lots $\times$ Blocks	25	29,723.9	1,189.0	
Total	215	371,293.9		

\*\* Significant at the 1 per cent point.

were no significant differences in the weight of fruits from plants of lots A and B and hence fresh and 8-month-old seed from the same source were equally good (Table IV, A vs. B).

Thirteen-year-old seeds of lot F and fresh and 8-month-old seeds (lots D and E) produced from lot F were all equal in value for use in field plantings (Table IV, F vs. D+E, and D vs. E). Thus the total variation in lots is to be accounted for by the poor yield of lot C.

#### LETTUCE

Lots B and D represent lettuce seeds stored for thirteen years in the laboratory and at  $-5^{\circ}$  C. respectively. Lots A and C represent fresh seeds produced from lots B and D and stored in the laboratory for about seven months before planting for field tests. Thus once again in this experiment we can see the effects of storage conditions of seeds of the same age upon the plants grown from them. Also we can compare the different ages of seeds from the same source.

A standard  $4 \times 4$  Latin square form served as a basis for field planting. Eight replications were made. Two lettuce plants were placed in each cell of the Latin squares. The measure of lettuce plant yields differed from those reported for aster, verbena, pepper, and tomato. In the four latter cases flowering and fruiting or sexual reproduction of the plants served for comparison. Since the commercial value of lettuce depends upon its vegetative growth, the size and weights of the heads were measured. These seeds were of the variety Iceberg. All of the plants were harvested on the same date, June 21, 1945. The entire plot was screened to prevent damage by rodents.

A summary of the yield in weight by blocks is presented in Table V and an analysis of variance may be seen in Table VI. There was a significant variation in lots, between Latin squares, in rows, and in columns. The cause of the variation of the lots is strikingly demonstrated. In this case the temperature at which the seeds had been stored for thirteen years was without effect on the plants produced from them. The age of the seeds, i.e., whether seven months old or thirteen years old, had a great effect and was in favor of the old seeds (see Table V). Also it will be noted that there was no interaction of storage temperature and age, i.e., the effect of age was the same for both conditions of storage.

#### DISCUSSION

All of the results reported above were for behavior of plants which had been grown from the variously treated seeds and which were established in the field. In only one case, i.e., that of tomato seeds which had been stored in an open container in the laboratory for thirteen years, did old seeds germinate to form plants which were inferior in the field. This is not to say that there were no differences in germination capacity of fresh and

TABLE V  
TOTAL WEIGHTS OF LETTUCE PLANTS IN OUNCES FOR SEED LOTS AND BLOCKS

Lot	Age of seeds	Block								Totals
		1	2	3	4	5	6	7	8	
A	7 mos.	69	76	75	75	56	72	62	75	560
B	13 yrs.*	76	88	93	73	66	86	77	82	641
C	7 mos.	76	87	61	75	58	69	79	60	574
D	13 yrs.†	80	104	77	66	67	83	91	90	658
Totals		301	355	306	289	247	310	309	316	2433

\* Stored with reduced moisture content in sealed containers in the laboratory.

† Stored with reduced moisture content in sealed containers at  $-5^{\circ}$  C.

old seeds of the various forms studied. That there were such differences is shown in Table VII. The particular lot of tomato seeds just mentioned gave only 6 per cent seedling production in soil in the greenhouse. This is to be compared with 85 to 97 per cent for all of the other lots including the ones stored for thirteen years under favorable storage conditions (lot F).

\* TABLE VI  
WEIGHTS OF LETTUCE PLANTS. ANALYSIS OF VARIANCE

Source of variation	D. F.	Sum of squares	Mean squares	F values
Lots				
Storage	1	3.75	3.75	0.57
Age	1	106.3	106.3	16.06**
Storage vs. Age	1	0	0	0
Blocks	7	197.0	28.14	4.25**
Rows	24	345.0	14.38	2.17*
Columns	24	419.0	17.46	2.64*
Replicate plants	128	806.0	6.30	0.95
Error within blocks	48	390.0	8.13	1.23
Lots $\times$ Blocks	21	139.0	6.62	
Total	255	2406.0		

\* Significant at the 5 per cent point.

\*\* Significant at the 1 per cent point.

Old pepper and verbena seeds showed reduced germination capacity when compared with fresh seeds (Table VII). Both fresh and old seeds of aster produced few seedlings in soil. In spite of these differences in ability to produce seedlings, there were no differences in the behavior of the resulting pepper, verbena, or aster seedlings in the field.

Old lettuce seeds from two storage temperatures gave 40 and 46 per cent germination as compared with 68 and 74 per cent germination of the fresh seeds (Table VII). It should be pointed out that the germination of

lettuce seed is reduced with high temperature such as prevailed for a time after planting all of these seeds. Consequently all of these figures are lower than was anticipated. In spite of their lower germination capacity, however, old seeds produced superior lettuce plants.

TABLE VII  
GERMINATION OF VARIOUS SEED LOTS WHEN THEY WERE SOWN  
TO PRODUCE SEEDLINGS FOR FIELD PLANTING

Variety	Per cent germination of seed lots of different ages					
	A	B	C	D	E	F
Aster	7 months	9.5 years	—	—	—	—
	15	35	—	—	—	—
Verbena	7 months	9.5 years	—	—	—	—
	51	13	—	—	—	—
Pepper	Fresh	8 months	13 years	—	—	—
	93	87	52	—	—	—
Tomato	Fresh	8 months	13 years*	Fresh	8 months	13 years**
	94	85	6	97	87	89
Lettuce	7 months	13 years†	7 months	13 years**	—	—
	68	40	74	46	—	—

\* Stored in open containers in the laboratory.

\*\* Stored with reduced moisture content in sealed containers at  $-5^{\circ}\text{C}$ .

† Stored with reduced moisture content in sealed containers in the laboratory.

The germination of the various lots of seeds has thus been shown to have no direct relation to the field performance of plants grown from them. However, if the viability of the seed is reduced too greatly, delay in germination followed by slow plant development decreases their value for the production of seedlings of good quality. This was seen for tomato seeds of lot C. Of course the actual number of plants produced from a batch of seeds as well as the quality of those plants is of great importance in commercial practice. If it is known, though, that good plants would be produced, rate of seeding could be adjusted to the known germination capacity. It should be kept in mind that the germination percentages from the old seeds were obtained directly upon removal from the storage condition. Experiments already reported (3, 5) for tomato, onion, and eggplant seeds have shown that deterioration in germination capacity of old seeds is rapid upon removal from favorable storage conditions to unfavorable ones.

In an earlier work it was pointed out that 6-year-old carrot, eggplant,

onion, tomato, and lettuce seeds produced normal plants but no quantitative data were taken (3). The same effect has been noted for delphinium plants produced from seeds stored 123 months (4).

There have been other reports from time to time on the value of old seeds for planting aside from their germination capacity. Rodrigo (12), working with mungo seeds, used 11- to 13-year-old seeds which had been stored in bottles sealed with paraffin at room temperature. As controls he used 1-month-old seeds taken from plantings of the old seeds previously made. At maturity the plants were harvested and data were taken on the number of pods produced, the weight of the dry pods, the weight of the straw, and the weight of the seeds per plant. In garden plots he obtained a significant difference in vine yields of new and old seeds in favor of the old seeds. Old seeds also gave a greater pod yield. The effect on the yield of beans was similar to that of the yield of pods except in one case where the pod and bean yield of new seeds was found to be slightly greater. He does not give germination percentages obtained for the various seeds used. He offers two possible explanations: (a) the vigor of the seed expressed in yielding power is correlated with its inherent longevity; or (b) the seed requires certain "seasoning" or "curing" before it attains the peak of its vitality.

Leus (10) studied the relation of farm crop seeds to production and found that in wet season culture in the Philippines the corn ear and mungo seed yield was greater from 13-month-old than from 3-month-old seed but in the yields of seed from cowpea and soybeans the reverse was true. In dry season crop also the oldest corn seeds (32 months old) resulted in the highest production of ears to the hill, but cowpea, mungo, and soybean plants from seeds eight months old gave a higher production than those from seeds 18 months old.

Bartlett in 1858 (1, p. 332) quoted from the Irish Farmers' Gazette which reported that, "The gardener knows that melon and cucumber seeds, if used of the last year's saving, produce plants too vigorous to produce much good fruit; whereas, those kept over for several years produce less rambling, but very fruitful plants."

Ohga (11) found that the length of sprouts from Indian lotus seeds at least 150 years old were always greater than that of sprouts from new seeds.

A note (8) on trials to determine the relative values of pumpkin seeds of different ages revealed that 3-year-old seed gave the best results in one trial but in another test 4-year-old seeds yielded less quantity but better quality pumpkins than 1-year-old seeds. It is not stated whether the yield was from a given number of seeds or a given number of plants.

Beattie, Jackson, and Currin (6) determined the yield of peanuts from old seeds. They did not measure yield from the same number of plants in all cases but rather yield from plants produced from a given number of



seeds regardless of the germination percentage. They stated (6, p. 9-10), "It will be noted that rather wide differences in percentage of germination, and hence in the stand from different lots of seed, in a single year were often accompanied by no appreciable differences in yield. These results may be accounted for in part by soil heterogeneity and in part by the larger yields of plants adjacent to missing hills. But the fact that yields obtained from poor stands sometimes equaled those from seed having a higher germination should not be taken as an indication that the use of poor seed is a profitable practice."

Kiesselbach (9) used corn seeds of various ages and tested the yield obtained. He found that satisfactory results may be expected from well matured viable seed corn aged up to four years, provided it has been kept free from external moisture and insect or rodent injury. He tested seeds from one to four years of age annually for five years. No significant differences were obtained in comparative acre yield in bushels from seeds of various ages. Also there were no material effects of seed age upon the vegetative development of the crop. He stated (9, p. 3), "Taking all things into consideration, new seed is to be preferred if its adaptation and viability are equal to the old seed. But it would seem preferable to use old seed of known local adaptation, rather than use new imported seed with which there has been no previous experience."

Crocioni (7) conducted soil experiments with *Triticum*, *Brassica*, *Medicago*, and *Trifolium pratense*, comparing seeds of different ages and following the development of the plants in each case. Plants from old seeds were retarded in growth and less resistant to adverse conditions. Paralleling the age of the seed, he found the plants were smaller and produced less throughout in wheat and *Brassica*. In the legumes the decreased production was less evident and was marked only in case of very old seeds.

The data presented in the present paper would certainly justify the use of old seeds within rather wide age limits. Normal seedlings were produced from old seeds of verbena and pepper. Likewise, old tomato seeds properly stored did not affect the plants grown from them. Superior performance of plants from old seeds of aster was evidenced by earliness of flowering. Similarly old lettuce seeds produced more desirable plants than fresh seeds. Plants from tomato seeds reduced to 6 per cent germination by improper storage conditions were inferior in quality. This indicates that there is a limit to the successful use of old seeds. That limit is imposed by inherent seed characteristics and the conditions under which they are held.

It should be pointed out that the summer of 1945 was a poor growing season. There was a great deal of rain and the temperature was low from May 29 to June 7. Behavior of seedlings in the field might vary with different weather conditions.

## GAS CONTENT OF PLANT TISSUE AND RESPIRATION MEASUREMENTS

F. E. DENNY

The aerobic respiration of plants consists fundamentally in the absorption of oxygen and the production of carbon dioxide. It is often assumed, therefore, that the rate of respiration could be measured either by determining the rate of decrease of oxygen or the increase of carbon dioxide in the air surrounding the tissue. This would be true, of course, only if there had been no change in the amount of the gas in question within the tissue during the period of the test. A recognition of the fact that the gas content of the tissue needs to be taken into account in respiration studies seems to have been made at an early date. Thus, in 1885 Dehérain and Maquenne (3, 4) in a study of the respiration of leaves refer to an *apparent* respiration (change in the amount of CO<sub>2</sub> in the air surrounding the leaves) and a *true* respiration (total change in CO<sub>2</sub> both in the air surrounding the leaves and in the gas removable from the leaves by subjecting the leaves to a vacuum). That the idea had an even earlier origin is shown by their reference to the views of Boussingault, who maintained that a true measurement of the gas exchange between the leaf and the surrounding atmosphere could be obtained only by taking into account the *atmosphere of the leaf* itself.

Although, therefore, it has been known, or at least thought, for nearly a century that the gas content of a tissue may be an important factor in obtaining an estimate of its respiration rate, not much was done about it until recently, and even now the field has not attracted many workers.

The main interest in internal gases has been in connection with fruits and vegetables in storage. The advances in this line were initiated by Magness (6) who developed a vacuum method of removing the gases from such tissue. This consisted in immersing the tissue in mercury in a gas-tight glass tube, applying a Torricellian vacuum, collecting the gas which escaped and transferring it to a gas-analysis apparatus. Various modifications were made by later workers. The object of these experiments was not to obtain a correct measurement of respiration rate, but to learn the gaseous environment of the internal cells under various conditions of storage, and to note whether the relative percentages of O<sub>2</sub> and CO<sub>2</sub> were related to certain disorders exhibited by the stored tissues. Only small amounts of tissue were used in such experiments until 1936 when Culpepper, Moon, and Lutz (1) reported the use of an enlarged apparatus with a volume of about 500 cc., but, of course, in such a case a correspondingly larger volume of mercury must be employed.

The most important recent advance is that of Whiteman and Schomer (11) who report the complete removal of the internal gas from sweet potato roots by vacuum. Gas extractions of tissue were carried out at the beginning and the end of the experimental period, and since the change in the gas content of the air surrounding the tissue in the closed containers was determined simultaneously, the true respiration rate was arrived at by taking into account the two components: change in air plus change in tissue. Such corrections were carried out for the carbon dioxide values; the oxygen values, however, are given only for the tissue itself, those for the surrounding air not being reported in the paper. Unfortunately, also, the authors do not describe the method or apparatus used in the vacuum extraction process, this being promised for a later paper by the junior author.

The above comments refer to the problem of removing the gas from the tissue and measuring its volume and composition with regard to oxygen, carbon dioxide, and nitrogen. The other problem, that of determining the carbon dioxide content alone and ignoring the other components, seems to have been solved satisfactorily. Papers which have attracted much attention and which emphasized the need of taking into account the  $\text{CO}_2$  content of the tissue in respiration studies were those of Willaman and coworkers (12, 13). They devised a method for determining the  $\text{CO}_2$  content of tissue, which consisted of placing the tissue in 95 per cent alcohol and aerating out the  $\text{CO}_2$  at the temperature of boiling alcohol, the  $\text{CO}_2$  being caught in alkali and estimated by titration. Wardlaw and Leonard (10) modified this method somewhat and found it accurate and useful. Some tests made in the present experiments substantiate this view. However, a somewhat more convenient method which obviated the necessity of using alcohol and making provision for a steam-bath for heating the alcohol was found. It consisted in disintegrating the tissue in a Waring blender in the presence of alkali, transferring the tissue pulp to an apparatus arranged for aeration in a current of air, liberating the  $\text{CO}_2$  by acidifying with  $\text{H}_2\text{SO}_4$ , aerating at room temperature, absorbing the  $\text{CO}_2$  in  $\text{Ba}(\text{OH})_2$ , and back-titrating. These two methods gave nearly equal estimates of the total  $\text{CO}_2$  in several tissues, and the decision as to which method is the more accurate and suitable under various conditions must be left for further experience.

The object of the present experiments was to obtain a complete extraction of the internal gas from relatively large quantities of tissue, at least 500 g. if the gas content was low, and to develop a routine procedure so that several samples of tissue could be run simultaneously. Since it did not seem feasible to use such large quantities of mercury as would be necessary under these conditions, a saturated solution of sodium chloride was used instead of mercury. As a source of vacuum, an ordinary water jet pump was

used and was found to develop a vacuum in the extraction vessel to the extent of at least 29 inches of mercury. The method was applied to several different kinds of plant tissue, these being chosen to represent types with both high and low amounts of gas contents. The results indicated that both oxygen and nitrogen were removed satisfactorily, but that carbon dioxide was not. Even after evacuation for one and one-half hours at 29 inches of mercury, or more, the tissue at the end could be shown to contain considerable quantities of  $\text{CO}_2$ ; indeed, in some cases the vacuum removed hardly more than 5 to 10 per cent of the  $\text{CO}_2$  that could be shown to be present by other methods. It is not believed that this failure is due to the particular type of extraction apparatus used in these tests, but that the difficulty is inherent in the vacuum method. Consequently it seemed that in obtaining a measure of the gas content of tissue, the oxygen and carbon dioxide should be estimated separately, oxygen by vacuum extraction, and carbon dioxide by aeration from the tissue into standard alkali.

These methods were applied in an experiment on the rate of respiration of potato tubers. The tubers were transferred from storage at  $5^\circ \text{C}$ . to the respiration test room at  $20^\circ \text{C}$ ., a procedure favorable for obtaining a high respiration rate. Samples of tubers were then enclosed in desiccators in which the tubers remained for intervals of 20, 44, 92, and 142 hours. Analyses of the air surrounding the tubers in the desiccators, and of the gas content of the tissues at the start and at the end of each interval, permitted the computation of the amount of  $\text{CO}_2$  formed and the amount of  $\text{O}_2$  used up. No experimental difficulties were encountered and the values obtained were consistent.

Evidence was obtained indicating that in determining the  $\text{CO}_2$  content of tissue in connection with the measurement of respiration rate the values found by the aeration method should be used, and not those found by the vacuum method which is believed not to be capable of removing the  $\text{CO}_2$  completely from the tissue, nor of giving a true picture of the changes in the  $\text{CO}_2$  content of such tissue.

In the present tests on the respiration of potato tubers a knowledge of the  $\text{O}_2$  content of the tissue was not of critical necessity, since nearly the same value for the  $\text{O}_2$  consumed in a given interval was obtained whether or not the internal  $\text{O}_2$  was taken into consideration. But the  $\text{O}_2$  content of potato tissue is relatively low, and the need of similar measurement with tissues containing larger amounts of  $\text{O}_2$  is clearly indicated.

#### APPARATUS AND METHODS

*Vacuum extraction of gas from plant tissue.* Figure 1 A shows the apparatus used in removing the gas from plant tissue. The tissue was weighed before and after wetting with water, and a note made as to the relation of these two weights. The object in wetting the surface of the tissue was to

avoid the enclosing of air bubbles which occurs when a dry tissue is put into water. The one-liter filter flask, Figure 1 A 1, was first partly filled with boiled, cooled, sodium chloride solution (approximately saturated NaCl at room temp.), and tilted so that the liquid came well up into the neck of the flask. The weighed tissue was cut into pieces small enough to allow them to enter the opening of the flask, and were pushed into the liquid one piece at a time, care being taken to avoid trapping any bubbles of air in depressions on the surface of the tissue (such as the eyes in potato tubers). This was continued until all of the tissue was added to the flask, which was then overflowing with liquid. Air bubbles at the top were skimmed off, and the rubber stopper was forced into place, the space at the top being examined for any trapped bubbles. The rubber stopper used was such that it could barely be forced into the flask. Pins were attached to the bottom of the stopper (see under stopper in flask in Fig. 1 A 2) in a circle around the tube, and these prevented any pieces of floating tissue from closing the exit. By adjusting the clamp and stopcock, Figure 1 A, 3 and 4, salt solution from the funnel, Figure 1 A 5, was passed into the flask until it overflowed into the vacuum extraction tube, 10 in Figure 1 A, care being taken that no air was trapped in the tubing at 6. The rubber tubing at 3, 6, and 8 were pieces of heavy-wall but flexible pressure tubing. Inside of the delivery tube at 7 was placed a two-inch packing of copper gauze made from one of the pads of copper commonly used in cleaning pots and pans; this screened out any bits of tissue and prevented clogging at the final exit tube in removing the sample of gas. The one-liter filter flask, Figure 1 A 9, was filled to the proper level with salt solution, and by applying suction at the exit, 8, the extraction tube, 10, was filled with salt solution and the clamp at exit, 8, was closed. With tissues containing air which is easily pulled out, e.g., apple or eggplant, it was found necessary to attach a screw-clamp temporarily on the tubing at 6, while the salt solution was being raised in the extraction tube. The vacuum was connected at Figure 1 A 11, and as air was removed from the tissue, the level of the salt solution in the extraction tube, 10, fell, and when this reached the lower portion of 10, the clamp, 11, was closed; after 5 to 30 minutes, depending upon the amount of gas coming from the tissue, the vacuum was disconnected, the clamp at 11 was cautiously opened and air allowed to enter the flask, 9; at the same time salt solution was allowed to flow into flask, 1, from funnel, 5, until finally the gas in 10 was at air pressure. It is then displaced by suction into a gas-sampling tube attached at 8. Inserting salt solution with a fine-pointed pipette into the rubber tube at 8, and manipulating with the fingers allows attaching the gas-sampling tube without introducing any air. Leaving the gas-sampling tube attached to 8, but closing the clamp at 8, the vacuum is again applied, and after a period of about 15 minutes another quantity of gas from the tissue is transferred

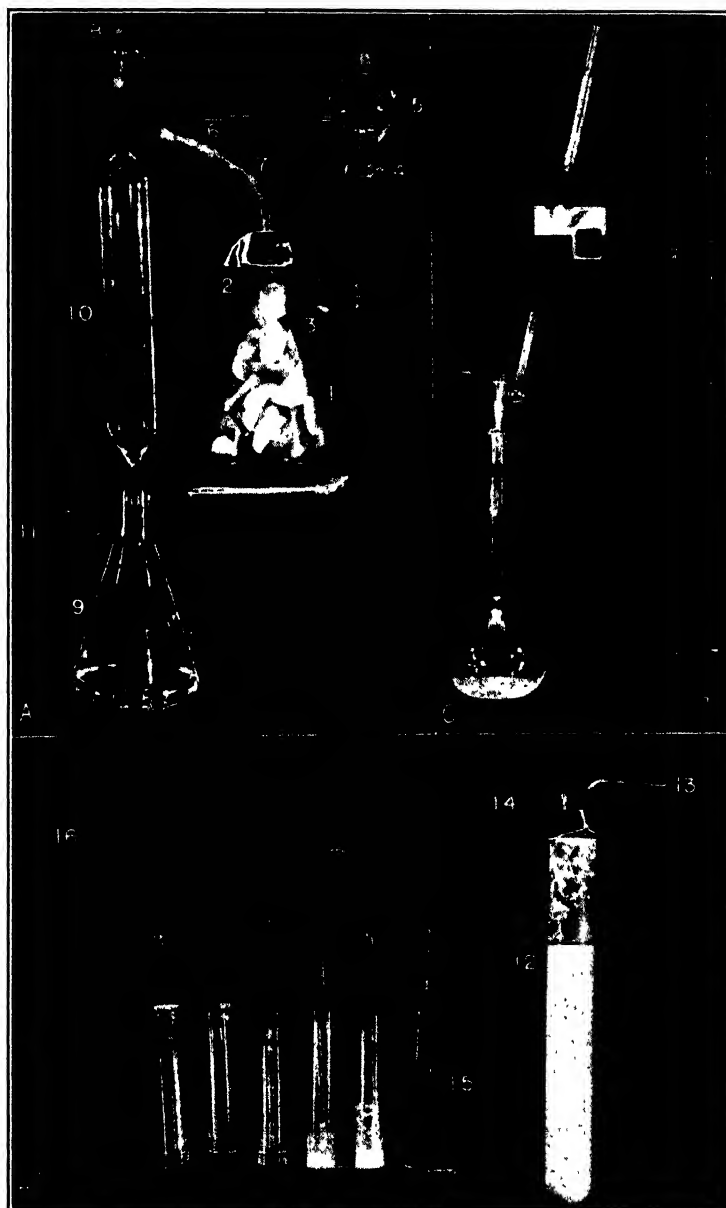


FIGURE 1. Apparatus used in estimating the  $O_2$  and  $CO_2$  contents of plant tissue. Details given in the text. A. Apparatus for removing gas from plant tissue by a vacuum. B. Apparatus for aerating  $CO_2$  out of plant tissue into test-tubes containing  $Ba(OH)_2$  solution. C. Method of transferring the  $Ba(OH)_2$  from the tubes in Figure 1 B 15 into a volumetric flask with a minimum of water, so that the aliquot factor in the back-titration may be as small as possible.

from 10 into the gas-sampling tube. This is continued until no gas, or only a small amount, is obtained after a suction-interval of 15 minutes. The sample of gas is then transferred to an Orsat gas-analysis apparatus. In the present experiments the burette of the Orsat apparatus had a volume of 100 cc. graduated to read 0.2 cc. The amounts of gas obtained from the tissues depended upon the kind and amount of tissue taken, varying in these tests from about 20 cc. to as much as 300 cc. When the amount of gas in a tissue was small, two tissue samples were taken and the gas removed from the two lots was combined in the transfer to the gas-analysis apparatus. The  $\text{CO}_2$  in the sample was absorbed in 20 per cent KOH, and the  $\text{O}_2$  with alkaline pyrogallol. The residual gas after  $\text{CO}_2$  and  $\text{O}_2$  were absorbed was assumed to be  $\text{N}_2$ .

*Aeration method for  $\text{CO}_2$  in plant tissue.* Into the bowl of a quart-size Waring blender (2) were poured 80 cc. of water and 60 cc. of an alkali solution made by dissolving 25 g. of NaOH in a liter of water. The plant tissue, cut into pieces, was dropped into this liquid, 2 cc. of capryl alcohol were added, the cover was placed on the blender and the machine was run until the tissue had been disintegrated. The cover was held in place for 20 seconds after the stirring had stopped and the liquid was poured into a large-size tall test-tube, 5 cm. internal diameter and 40 cm. tall, volume 650 to 700 cc. (see Fig. 1 B 12) the tissue adhering to the bowl being rinsed out with a solution made by adding 5 cc. of the alkali solution to 45 cc. of  $\text{H}_2\text{O}$ , the 50 cc. being used up in three rinsings. If the volume permits, further rinsings with water may be made. With potato tissue the pH at this stage was found to be about 10. The rubber stopper closing the tube, 12, was fitted with inlet and outlet tubes, the inlet tube, 13, with orifice previously reduced to about 2 mm. reaching to the bottom of the tall tube, and the outlet tube, 14, reaching only a few mm. below the rubber stopper. The outlet tube was then connected to the series of aeration tubes, Figure 1 B 15. A small funnel was connected to the inlet tube at 13, and, while a slow current of air was being pulled through the system, 60 cc. of a sulphuric acid solution (~~50~~ cc. of conc.  $\text{H}_2\text{SO}_4$  to sufficient water to make one liter after cooling) were poured in. The inlet tube, 13, was connected to a bubbler tube containing dilute  $\text{Ba}(\text{OH})_2$  solution (at right, not shown in the photograph) and this to a soda-lime tube to remove  $\text{CO}_2$  from the entering air. Spring clamps of the Fisher type were attached to the rubber tubing close to the places of connection to the inlet and outlet tubes, 13 and 14, and the tissue in the tube, 12, was thoroughly mixed by repeatedly upending and shaking it. At this stage with potato tissue the pH was found to be about 2. The spring clips were then removed and a current of air was drawn through the system by applying suction at 16.

This method of removing  $\text{CO}_2$  from an air-stream is that used by Miller (7, 8). The test tubes at 15 were 200 mm. long and 32 mm. outside diam-

eter. They are listed in laboratory supply catalogs as Van Slyke-Cullen urea tubes. The first tube at the right at 15 was a guard-tube, and the other five tubes contained the barium hydroxide solution used to absorb the  $\text{CO}_2$ . At first the air current was adjusted to a slow rate so that most of the  $\text{CO}_2$  would be caught in the first tube containing  $\text{Ba}(\text{OH})_2$ . The rate was then increased gradually until at the end a brisk rate of aeration was maintained. The progress of the absorption of  $\text{CO}_2$  could be followed by the amount of precipitate of  $\text{BaCO}_3$  formed in the series of tubes. When the amount of  $\text{CO}_2$  was such as to require more  $\text{Ba}(\text{OH})_2$  tubes the aeration was stopped, a spring clamp was attached to the rubber tube connecting the first  $\text{Ba}(\text{OH})_2$  tube and the guard-tube, and a fresh tube of  $\text{Ba}(\text{OH})_2$  solution was inserted in the series. At intervals the tissue in tube 12 was mixed by stopping the aeration and upending the tube in the manner indicated above. Nearly all of the  $\text{CO}_2$  is aerated over in the first half-hour, and after one hour only traces of  $\text{CO}_2$  are left. In these tests aeration was continued for one and one-half hours.

The  $\text{Ba}(\text{OH})_2$  solution was prepared by diluting 1:5 an approximately saturated solution (at room temperature), 25 cc. of the diluted solution being pipetted into each of the tubes. This allowed a rapid air current such as was used at the end of a test without undue splashing.

To those who feel that there is danger of loss of  $\text{CO}_2$  by absorption in the rubber connections from one tube to another in the aeration system, it may be pointed out that the rubber tubes may be replaced by U-tubes made of glass tubing fitting end to end with the aeration tubes, so that rubber tubing is used only to hold these tubes in place.

The amount of tissue that can be successfully disintegrated in the Waring blender under these conditions and then aerated smoothly has varied with different tissues, the essential requirement being that the tissue sample after acidification shall be a liquid which is not too viscous. For potato tuber 250 g. furnishes a good sample, while for gladiolus bulbs not more than about 150 g. could be used.

At the end of the aeration period, the liquid in the  $\text{Ba}(\text{OH})_2$  tubes was transferred to a volumetric flask. In doing this it should be remembered that the titration is to involve only the unused portion of the  $\text{Ba}(\text{OH})_2$  and that any precipitate of  $\text{BaCO}_3$  which adheres tightly to the sides of the test-tube need not be removed.

It is desirable to keep the volume of the liquid obtained by decanting and rinsing into the volumetric flask low, in order that the aliquot factor may be as small as possible. This point seems to be sufficiently important to justify a detailed description of the process of transferring the liquid to the volumetric flask. Figure 1 C shows the stage at which the liquid in the tube has been decanted into the volumetric flask. The bulb of the aeration tube has been suspended so that one of the holes in the bulb touches the



funnel in which case drainage will occur. The liquid remaining in the test-tube and in the aeration tube is then transferred to the funnel by a fine jet of distilled water from a water-bottle on a shelf above the laboratory table. The rubber-tube siphon from the water-bottle ends in a piece of glass tubing drawn out to produce a fine jet of water controlled by a Fisher-type pinch clamp. While the bulb is draining the test-tube is rinsed out by three successive washings with the jet of water, using about 4 cc. for each washing. Then the outside of the aeration tube is rinsed with about 4 cc. of water. The jet of water is then directed into the inside of the aeration tube and this is rinsed with three successive amounts of about 4 cc. each. Finally the aeration tube is again placed in the test-tube and the final rinsing made with about 5 cc. Rinsing the funnel completes the transfer. About 35 cc. of  $H_2O$  suffices to transfer the  $Ba(OH)_2$  from the test tube to the volumetric flask, and the time required is about 45 seconds for each tube.

The liquid in the volumetric flask was mixed and the precipitate was allowed to settle partially. Aliquots of 50 or 100 cc. were taken for titration against 0.1 N HCl, phenolphthalein being the indicator.

A control against which these values were compared was run each day simultaneously with the tissue samples, and was a repetition of the procedure just described except that no tissue was added to the alkali solution at the start.

*The alcohol method for  $CO_2$  in plant tissue.* The methods previously employed by Willaman and Brown (13) and by Wardlaw and Leonard (10) were employed in a modified form for use with some of the tissues in these experiments in order to get a comparison between the two methods for  $CO_2$ . In Figure 1 B instead of the tall test-tube at 12, a glass bottle approximately 1500 cc. volume with wide opening was used. The air inlet tube led to the bottom of the bottle at a place near the edge of the rubber stopper, and a reflex condenser 15 inches long with an 8-inch 4-bulb condensing portion was connected through the center of the stopper. A rubber stopper fitted with a bent glass tube led from the top of the condenser to the chain of  $Ba(OH)_2$  aeration tubes shown in Figure 1 B 15. The first tube in the series of six tubes was a guard tube, and prevented accidental splashing from the condenser. At the start of the test the plant tissue was cut into pieces, dropped into cold 95 per cent alcohol in the glass bottle, and by tilting the bottle sideways the stopper and the air inlet tube were fitted into place. The bottle containing the tissue in 95 per cent alcohol was then immersed in water in a steam-bath, and the heat turned on. The aeration continued as described in the previous paragraphs. Into the air current two T-tubes were inserted, one between the bottle and the bubbler from the soda-lime tube, and one between the bottle and the  $Ba(OH)_2$  tubes. These two tubes were equipped with short pieces of rubber tubing and

with spring clamps and served to furnish quick release of pressure or vacuum if any clogging of the system occurred. The temperature of the water bath was adjusted to give the proper rate of condensation of alcohol vapor. About one and one-half hours was sufficient to drive off the  $\text{CO}_2$  from the tissue sample. The control consisted of a repetition of this procedure, in all respects the same, except that no tissue was placed in the bottle containing the alcohol.

*Comparison of results by the two methods for  $\text{CO}_2$  in tissue.* A few tests were carried out in which tissue was divided into two equal portions and the  $\text{CO}_2$  content was estimated by each method. The results are shown in Table I. There seems to be no difference in the amounts found by the two

TABLE I  
COMPARISON OF TWO METHODS OF ESTIMATING THE  
TOTAL  $\text{CO}_2$  CONTENT OF TISSUE

Kind of tissue	Wt. of tissue, g.	Mg. $\text{CO}_2$ found	
		By disintegrating the tissue in alkali, acidifying, and aerating at room temp.	By putting tissue in 95% alcohol and aerating at the b.p. of alcohol
Potato	500	100	116
Potato	500	252	228
Potato	500	286	254
Turnip	398	57	58
Eggplant	215	22	20
Apple	455	40	48

methods. Further experiments would be needed with different tissues and with different  $\text{CO}_2$  contents to permit a choice between them. The alcohol method is preferable when the  $\text{CO}_2$  content of the tissue is very low, since larger amounts of tissue can be employed in a sample. If a special steam-bath were built, so that access to the apparatus from both sides of the bath could be had, the alcohol method would be about as convenient as the other.

*Plant tissues used.* The potato (*Solanum tuberosum* L.) tubers, variety Irish Cobbler, were grown in the Institute gardens in 1945, and had been stored at a temperature of  $5^\circ\text{C}$ . for several weeks previous to use in these tests. The gladiolus corms, variety Laughing Water, were of the 1945 Institute crop, and had been stored from harvest until sampling date in moist soil in flats at a temperature of approximately  $25^\circ\text{C}$ . The other fruits and vegetables were obtained from the local market, and except in the case of apple fruits, the name of the variety was not known. The materials used were as follows: fruits of eggplant (*Solanum melongena* L.), summer squash (*Cucurbita pepo* L.), and apple (*Pyrus malus* L.) varieties

Delicious, Winesap, and Baldwin, roots of turnip (*Brassica rapa* L.), and sweet potato (*Ipomoea batatas* Poir.).

## RESULTS

## GAS CONTENT OF VARIOUS TISSUES

The gas content of various tissues found by these methods of removing the gas are shown for various tissues in Tables II and III. In Table II the amounts of the different fractions of gas removable by the evacuation method are shown for half-hourly intervals in columns 4, 5, and 6. Most of the gas was removed during the first 30 minutes of vacuum (see column 4). By the end of one hour all of the oxygen extractable by this method had been removed and about 99 per cent of the nitrogen. The carbon dioxide

TABLE II  
GAS CONTENT OF PLANT TISSUES, SHOWING ESPECIALLY THE VOLUMES OBTAINED AT HALF-HOUR INTERVALS BY THE APPLICATION OF VACUUM

Kind of tissue	Wt. of tissue, g.	Kind of gas	Cc. of gas at prevailing temperature and pressure				
			Vol. by vacuum at intervals of			Total by vacuum	CO <sub>2</sub> removed by aeration method
			1st 30 min.	2nd 30 min.	3rd 30 min.		
Potato	1014	Total	23.6	1.6	0.6	25.8	—
		CO <sub>2</sub>	2.0	0.2	0.0	2.2	121
		O <sub>2</sub>	3.6	0.0	0.0	3.6	—
		N <sub>2</sub>	18.0	1.4	0.6	20.0	—
Potato	1037	Total	36.6	4.1	4.6	45.3	—
		CO <sub>2</sub>	16.4	3.4	4.3	24.1	288
		O <sub>2</sub>	0.9	0.0	0.0	0.9	—
		N <sub>2</sub>	19.3	0.7	0.3	20.3	—
Turnip	866	Total	115.2	15.4	0.0	130.6	—
		CO <sub>2</sub>	4.0	2.8	0.0	6.8	68
		O <sub>2</sub>	19.4	0.2	0.0	19.6	—
		N <sub>2</sub>	91.8	12.4	0.0	104.2	—
Eggplant	424	Total	289.3	13.2	2.4	304.9	—
		CO <sub>2</sub>	4.9	2.9	1.7	9.5	25
		O <sub>2</sub>	55.8	0.0	0.0	55.8	—
		N <sub>2</sub>	228.6	10.3	0.7	239.6	—
Squash	183	Total	38.6	1.5	3.4	43.5	—
		CO <sub>2</sub>	4.7	1.0	2.8	8.5	17
		O <sub>2</sub>	6.3	0.0	0.0	6.3	—
		N <sub>2</sub>	27.6	0.5	0.6	28.7	—
Apple var. Delicious	902	Total	206.8	6.7	4.6	218.1	—
		CO <sub>2</sub>	16.6	3.1	3.0	22.7	43
		O <sub>2</sub>	29.1	0.4	0.0	29.5	—
		N <sub>2</sub>	161.1	3.2	1.6	165.9	—

Note: N<sub>2</sub> by difference.

was removed more slowly and less completely, there being about 85 per cent of the total extractable by this method removed at the end of one hour.

The small amounts of gas shown in column 5 and 6 of Table II, i.e., volumes of a few tenths of a cc., or even of 1 to 5 cc., can not, of course, be estimated directly in the gas analysis apparatus used, not because such volumes can not be measured but because they can not be brought into contact successfully with the reagents in the absorbing pipettes. In order to obtain better readings in such cases when it was known that the reading would be low, the residual gas from the preceding test was pushed back into the pyrogallol pipette and held there as a reserve. Then the small quantity of gas to be estimated was brought into the measuring burette, the volume read, the residual gas from the pyrogallol pipette brought over and added to the gas sample, and the volume of combined lots read. After the absorption in the Orsat pipettes the proper deduction for the residual added gas was made.

The total amounts of gas extractable by vacuum from the various tissues are shown in column 7 in Table II, the values given being for the weight of tissue taken (see column 2). Computed on the kilogram basis the total gas varied from about 25 cc. for potato tissue to about 700 cc. for eggplant;  $\text{CO}_2$  varied from 2 cc. per kg. for potato to 46 cc. for squash;  $\text{O}_2$  from 1 cc. for potato to 113 cc. for eggplant. The apparatus operated successfully within these ranges of gas content.

The turnip, eggplant, squash, and apple tissues were taken as found in the sales bins at the market, but the two potato lots in Table II were chosen to represent different conditions of storage; the tubers for the lot first listed in Table II were from bags of tubers stored for several weeks at  $5^\circ \text{C}$ ., while the lot listed second were tubers that were removed from storage at  $5^\circ \text{C}$ . and were placed in a desiccator which was closed overnight to allow an accumulation of  $\text{CO}_2$ . The analyses of the internal gas found in these two lots show the effect of the conditions previous to the time of sampling.

The values in column 8 in Table II are those obtained on the same day from the same tissues when the  $\text{CO}_2$  was estimated by the aeration method, i. e., by disintegrating the tissue in dilute alkali, acidifying with  $\text{H}_2\text{SO}_4$ , and aerating out the total  $\text{CO}_2$  into  $\text{Ba}(\text{OH})_2$  and back-titrating. A comparison of the  $\text{CO}_2$  values in column 7 with those for  $\text{CO}_2$  in column 8 show that usually only a small part of the total  $\text{CO}_2$  of the tissue could be removed by the application of the vacuum. With potato only 10 per cent or even less of the total  $\text{CO}_2$  shown by the aeration method was removable by vacuum, while with squash and apple the proportion was about 50 per cent.

After it was found that an evacuation period of one hour was sufficient

to obtain about all of the gas that could be removed from the tissue in that manner, the experiments were continued by combining the gas extractions up to the end of one hour, and then analyzing the accumulated total in the Orsat apparatus. The results are shown in Table III, and along with them are given the corresponding total CO<sub>2</sub> values obtained

TABLE III

VOLUMES OF GAS REMOVED FROM VARIOUS TISSUES BY VACUUM FOR ONE HOUR, OR BY AERATION OF TISSUE AFTER DISINTEGRATION IN DILUTE ALKALI AND ACIDIFYING

Tissue and wt. of sample	Kind of gas	Cc. of gas obtained		Tissue and wt. of sample	Kind of gas	Cc. of gas obtained	
		By vacuum	By aeration			By vacuum	By aeration
Gladiolus, 500 g.	Total	19.4	—	Sweet potato, 300 g.	Total	37.4	—
	CO <sub>2</sub>	0.6	13		CO <sub>2</sub>	3.6	39
	O <sub>2</sub>	4.2	—		O <sub>2</sub>	6.2	—
	N <sub>2</sub>	14.6	—		N <sub>2</sub>	27.6	—
Gladiolus, 496 g.	Total	23.8	—	Sweet potato, 300 g.	Total	51.2	—
	CO <sub>2</sub>	7.3	51		CO <sub>2</sub>	8.2	26
	O <sub>2</sub>	2.5	—		O <sub>2</sub>	8.0	—
	N <sub>2</sub>	14.0	—		N <sub>2</sub>	35.0	—
Eggplant, 180 g.	Total	100.4	—	Apple, Baldwin, 350 g.	Total	110.0	—
	CO <sub>2</sub>	3.6	6		CO <sub>2</sub>	9.4	10
	O <sub>2</sub>	18.0	—		O <sub>2</sub>	17.0	—
	N <sub>2</sub>	78.8	—		N <sub>2</sub>	83.6	—
Apple, Winesap, 310 g.	Total	74.2	—	Potato, 1000 g.	Total	28.2	—
	CO <sub>2</sub>	11.2	18		CO <sub>2</sub>	3.0	80
	O <sub>2</sub>	6.0	—		O <sub>2</sub>	5.6	—
	N <sub>2</sub>	57.0	—		N <sub>2</sub>	19.6	—
Eggplant, 155 g.	Total	99.8	—	Potato, 1000 g.	Total	41.8	—
	CO <sub>2</sub>	1.8	3		CO <sub>2</sub>	20.4	203
	O <sub>2</sub>	19.4	—		O <sub>2</sub>	1.4	—
	N <sub>2</sub>	78.6	—		N <sub>2</sub>	19.0	—

Note: N<sub>2</sub> by difference. Gas volumes at the prevailing temperature and barometric pressure. Volumes by vacuum method measured by burette, volumes by aeration method computed from titration values.

with the aeration method for CO<sub>2</sub>. The eggplant, apple, and sweet potato tissues were used in the condition in which they happened to be at the time of purchase, but the gladiolus corms and potato tubers had had different previous histories at the time of sampling. The gladiolus corms were removed from the soil in flats in which they had been stored in moist soil from October 1945 to March 1946. They were still dormant. The lot first listed in Table III was analyzed at once, while the lot listed second in Table III was placed in a desiccator, the cover was put on, and the corms allowed to accumulate CO<sub>2</sub> overnight. A similar situation occurred with the potato samples in Table III, the first listed lot being tubers taken from

storage at 5° C., the second after such tubers had accumulated CO<sub>2</sub> in a closed desiccator.

Here again, the amount of CO<sub>2</sub> found by the aeration method was usually greater, and often much greater than that found by evacuation. The difference was less extensive with apple, and probably non-existent in the Baldwin sample, but with sweet potato, and particularly with potato, the CO<sub>2</sub> values obtained by vacuum were much lower than those by aeration.

Evidence is presented in the next section to indicate that for purposes of estimating the rate of CO<sub>2</sub> production in respiration experiments the CO<sub>2</sub> values by the aeration method are the ones that should be employed and not those obtained by vacuum extraction.

#### APPLICATION OF THESE METHODS FOR THE GAS CONTENT OF TISSUES TO A RESPIRATION EXPERIMENT

In order to test these methods of determining the gas content of tissue in routine analyses of tissue, and to note what information they would bring to the problem of measuring rate of respiration, an experiment on the respiration of potato tubers was carried out.

Tubers were removed from a cold storage room at 5° C., were divided into five equal lots of 1750 g. each, and were spaced out on a table in a constant temperature room until they had attained the temperature of the room (which was at 20° C. and was maintained so during the experiment). One lot of tubers was taken for analysis at the start of the test, and the other lots were placed in large desiccators each with known volume, also previously brought to the temperature of the room, and each fitted with a glass tube leading to the center in order to provide for removing samples of the air surrounding the tubers at the end of the test. One lot was removed at the end of 20 hours, one after 44 hours, one after 92 hours, and one after 142 hours.

Analyses of samples of gas taken from the desiccators at the end of each interval were carried out and computations were made by the method of Haldane and Graham (5, p. 56); these together with data on the temperature, barometric pressure, change in gas pressure within the desiccator during the interval, and volumes of desiccators and tuber samples permitted computation of the changes in the amounts of CO<sub>2</sub> and O<sub>2</sub> between the start and end of each interval. The composition of the gas samples is shown in Table IV, and the changes found in the amounts of CO<sub>2</sub> and O<sub>2</sub> are given in lines 1 and 4 in Table VI.

Tubers were taken at the start of the test and at the end of each of the time intervals for estimates of the amounts of internal gases by the vacuum method for CO<sub>2</sub>, O<sub>2</sub>, and N<sub>2</sub>, and by the aeration method for CO<sub>2</sub>. The results are shown in Table V. The differences in the amounts of CO<sub>2</sub>

and  $O_2$  in the tissue during the intervals are computed from Table V and are shown in lines 2, 3, and 5 in Table VI. The  $CO_2$  content of the tissue whether measured by vacuum extraction or by aeration, see lines 2 and 5,

TABLE IV  
PERCENTAGE COMPOSITION OF THE AIR SURROUNDING  
THE TUBERS IN THE CONTAINERS

Kind of gas	Per cent by volume				
	At start	After 20 hours	After 44 hours	After 92 hours	After 142 hours
$CO_2$	0.0	5.5	11.5	18.4	21.9
$O_2$	20.9	11.6	4.1	0.0	0.0
$N_2$	79.1	82.9	84.4	81.6	78.1

Note:  $N_2$  by difference.

Table V, showed increases during the progress of the experiment, but there was a great divergence between the two methods as to the amounts indicated as being present. The amount shown by evacuation was only about 5 to 20 per cent of that found by aeration. Only a small amount of oxygen (8.2 cc. in 1750 g. of tissue) was found at the start (see line 3, Table V), and this amount decreased to 1.4 cc., but did not fall to zero as might be expected, since Table IV shows that shortly after 44 hours no oxygen was

TABLE V  
GAS CONTENT OF TUBERS AT THE START AND AT THE END OF  
EACH EXPERIMENTAL INTERVAL

Method	Kind of gas	Cc. at 20° and 760 mm. in 1750 g. of tissue				
		At start	After 20 hours	After 44 hours	After 92 hours	After 142 hours
Removed by vacuum	Total	49.0	94.5	128.1	107.7	132.5
	$CO_2$	7.0	60.7	91.7	75.0	100.1
	$O_2$	8.2	2.1	2.8	1.4	1.4
	$N_2$	33.8	31.7	33.6	31.3	31.0
Removed by aeration	$CO_2$	158	347	451	498	503

Note:  $N_2$  by difference. Values by vacuum method by burette measurements, values by aeration method by computation from titration values.

present in the desiccators. The small amount found at the 92-hour interval and later, was undoubtedly introduced into the tissue during the process of preparing the tissue sample, and putting the cut tissue into the extraction flask. Indeed, it is noteworthy that more oxygen was not introduced in this way, since presumably the tissue was oxygen-free when removed

from the desiccators, and then cut tissue was exposed at least for a few seconds to air containing 20.9 per cent oxygen.

The total gas changes in the air surrounding the tissue and in the tissues themselves during each one of the intervals in the experiment are shown in Table VI. From the data in Table VI one may make various combinations of the values to compute respiration rates and respiration ratios (volume of  $\text{CO}_2 \div$  volume of  $\text{O}_2$ ). For example, what is believed to be the true respiration rate for the potato tubers during the interval 0 to 20

TABLE VI  
CHANGES IN  $\text{CO}_2$  AND  $\text{O}_2$  IN THE SURROUNDING AIR AND IN THE TISSUES  
OF THE POTATO TUBER IN THE RESPIRATION TEST

Changes measured	Change in cc. of gas at 20° C. and 760 mm. in 1750 g. of tubers, or in air surrounding the tubers, during the following intervals			
	0 to 20 hours	0 to 44 hours	0 to 92 hours	0 to 142 hours
Increase in $\text{CO}_2$ in air surrounding the tubers	234	467	713	1070
Increase in $\text{CO}_2$ in tissue as measured by the aeration method	189	293	340	345
Increase in $\text{CO}_2$ in tissue as measured by the vacuum method	54	85	68	93
Decrease in $\text{O}_2$ in air surrounding the tubers	439	740	811	905
Decrease in $\text{O}_2$ in tissue as measured by the vacuum method	6	5	7	7

hours (see column 2, Table VI) is computed as follows (volumes of gas at 20° C. and 760 mm.):

$$\text{CO}_2 \text{ increase in air surrounding tubers} = 234 \text{ cc.}$$

$$\text{CO}_2 \quad \text{"} \quad \text{"} \quad \text{tissue} = 189 \text{ "}$$

$$\text{Total CO}_2 \text{ produced} = 423 \text{ "}$$

$$\text{O}_2 \text{ decrease in air surrounding tubers} = 439 \text{ cc.}$$

$$\text{O}_2 \quad \text{"} \quad \text{"} \quad \text{tissue} = 6 \text{ "}$$

$$\text{Total O}_2 \text{ used up} = 445 \text{ "}$$

$$\text{CO}_2 \text{ produced} = \frac{423}{1.75 \times 20} = 12.1 \text{ cc. CO}_2/\text{kg.}/\text{hr.}$$

$$\text{O}_2 \text{ consumed} = \frac{445}{1.75 \times 20} = 12.7 \text{ cc. O}_2/\text{kg.}/\text{hr.}$$

$$\text{Ratio CO}_2 \div \text{O}_2 = 423 \div 445 = 0.95.$$



If now, instead of using the values for  $\text{CO}_2$  in the tissue by the aeration method, those for the vacuum method are employed, the total  $\text{CO}_2$  produced was  $234 \text{ cc.} + 54 \text{ cc.} = 288 \text{ cc.}$ , giving the following computations:

$$\text{CO}_2 \text{ produced} = \frac{288}{1.75 \times 20} = 8.2 \text{ cc. CO}_2/\text{kg./hr.}$$

$$\text{O}_2 \text{ consumed} = \frac{445}{1.75 \times 20} = 12.7 \text{ cc. O}_2/\text{kg./hr.}$$

$$\text{Ratio CO}_2 \div \text{O}_2 = 288 \div 445 = 0.65.$$

The so-called "apparent" respiration, i.e., change in  $\text{CO}_2$  and  $\text{O}_2$  in the air surrounding the tissue, would give the following values:

$$\text{CO}_2 \text{ produced} = \frac{234}{1.75 \times 20} = 6.7 \text{ cc. CO}_2/\text{kg./hr.}$$

$$\text{O}_2 \text{ consumed} = \frac{439}{1.75 \times 20} = 12.5 \text{ cc. O}_2/\text{kg./hr.}$$

$$\text{Ratio CO}_2 \div \text{O}_2 = 234 \div 439 = 0.53.$$

There was some oxygen still available to the tubers at the end of the 44th hour, and computations for the interval 0 to 44 hours are as follows: the  $\text{CO}_2$  produced  $= 467 + 293 = 760 \text{ cc.}$ , and the  $\text{O}_2$  consumed  $= 740 + 5 = 745 \text{ cc.}$  The rate of respiration on the basis of the  $\text{CO}_2$  produced was  $760 \div (1.75 \times 44) = 9.9 \text{ cc. per kg. per hour}$ , and the values for  $\text{O}_2$  consumed were  $745 \div (1.75 \times 44) = 9.7 \text{ cc. per kg. per hour}$ . The ratio of  $\text{CO}_2$  to  $\text{O}_2$  (by volume) was  $760 \div 745 = 1.02$ . If, however, the computation had been made on the basis of the  $\text{CO}_2$  found in the tissue by the vacuum method, the  $\text{CO}_2$  produced would have been  $467 + 85 = 552 \text{ cc.}$ , which would have indicated a respiration rate of  $7.2 \text{ cc. CO}_2/\text{kg./hr.}$ , and a respiratory ratio of  $552 \div 745 = 0.74$ .

If computations are made for the intervals 0 to 92 hours, and 0 to 142 hours, it should be kept in mind that during a considerable part or nearly all of such intervals, the respiration was anaerobic. Values horizontally in lines 1 and 4 in Table VI are not strictly comparable since the volumes of the desiccators were not exactly the same, but horizontal differences in lines 2, 3, and 5 are comparable, as are all values vertically in all columns.

It will be noted that the oxygen content of the tissue was not an important factor in computing the true  $\text{O}_2$  respiration. The change in  $\text{O}_2$  in the tissue was so small, as compared with the change in  $\text{O}_2$  in the air surrounding the tissue, that the correction applied for  $\text{O}_2$  in the tissue had little effect. Thus, the "true"  $\text{O}_2$  respiration in the interval 0 to 20 hours was 12.7, and the "apparent"  $\text{O}_2$  respiration (ignoring the change in  $\text{O}_2$  in the tissue) was 12.5 cc./kg./hr.

## DISCUSSION

In attempting to compare the results for internal gas obtained with the apparatus used in these tests with those reported by previous workers using the Magness apparatus, or modifications of it, it was found that nearly all of these workers reported the percentage of  $\text{CO}_2$  and  $\text{O}_2$  in the extracted gas but not the volume which was obtained. Smith (9), however, gave the volume obtained from potato tissue in a number of cases, as, for example, 5.2 to 7.8 cc. of total gas in 175 g. of tissue, 4.3 to 5.3 cc. in 180 g., 3.6 to 4.5 cc. in 172 g., 4.2 to 5.7 cc. in 180 g. These show a range from about 24 to 45 cc. of gas per kilogram of tissue. The values in the present experiment for potato tuber tissue as shown in Tables II and III are about 25 to 45 cc. per kg. of tissue. Culpepper *et al.* (1) reported that the total extractable gas obtained from eggplant tissue was 15 to 30 per cent of the volume of the fruit. The volume of 500 g. of eggplant tissue was estimated in the present tests to be about 900 cc. which indicates a gas content of 135 to 270 cc. of total gas in 500 g. of tissue in their experiment. The values obtained in the present tests for eggplant as shown in Tables II and III vary from 280 to 360 cc. per 500 g. of tissue. Whiteman and Schomer (11) used sweet potato roots, and if the values for their control roots at the start of the experiment and for their "open basket" lots are combined and averaged, their method extracted about 200 cc. of total gas, 56 cc. of  $\text{CO}_2$ , 25 cc. of  $\text{O}_2$ , and 119 cc. of  $\text{N}_2$  from a kg. of tissue. Two lots of sweet potatoes were extracted in the present tests, and the values per kg. obtained shown in Table III were as follows: in one test, total = 124 cc.;  $\text{CO}_2$  = 12 cc.;  $\text{O}_2$  = 20 cc.;  $\text{N}_2$  = 92 cc. In the other test, total = 171 cc.;  $\text{CO}_2$  = 27 cc.;  $\text{O}_2$  = 27 cc.;  $\text{N}_2$  = 117 cc. The differences are mainly due to the amount of  $\text{CO}_2$  found, and this constituent is subject to much variation in tissues depending upon the conditions previous to sampling. The  $\text{O}_2$  and  $\text{N}_2$  values agree satisfactorily with those of Whiteman and Schomer, and especially so for the second sweet potato sample.

With regard to the  $\text{CO}_2$  obtained from plant tissues in these experiments, and especially in the cases in which the aeration method was used, it is not known in what form these amounts of  $\text{CO}_2$  may occur in the tissues. Presumably only a small part is present as a free gas in the intercellular spaces, much of it no doubt in solution in the plant liquids, and possibly some of it as bicarbonate, carbonate, or in some other combination with tissue constituents. The acidity furnished by the  $\text{H}_2\text{SO}_4$  would be expected to liberate  $\text{CO}_2$  from any of these combinations. The situation with respect to the alcohol method is not clear on this point, as no acid is added previous to aeration. Possibly the natural acidity of the tissue is sufficient to effect the liberation of  $\text{CO}_2$  from some or all of such combinations as may exist.

At the time these experiments were started it was recognized that a

knowledge of the  $\text{CO}_2$  content of the tissue was necessary before an accurate measurement of the  $\text{CO}_2$  output of a tissue could be reached, and it was assumed that this was true also of the  $\text{O}_2$  content; that until the change in  $\text{O}_2$  in the air surrounding the tissue had been corrected by an estimate of the change in  $\text{O}_2$  in the tissue during the same interval, the true amount of  $\text{O}_2$  consumed could not be known; in other words, that the mere change in  $\text{O}_2$  in the air surrounding the tissue would not be sufficiently accurate for an estimate of the  $\text{O}_2$  respiration. The present experiments have made necessary a modification of this view, so far as potato tissue is concerned. Computing the  $\text{O}_2$  consumption merely by the change in  $\text{O}_2$  in the surrounding air without regard to the  $\text{O}_2$  content of the tissue would have given an acceptable value in this experiment. The need of investigations on other tissues from this point of view, especially with tissues containing relatively large amounts of  $\text{O}_2$  is apparent.

An interesting feature in Table V is the relative constancy of the nitrogen values in line 4. Although the total quantity of vacuum-extractable gas varied from 49.0 to 132.5, and the amount of  $\text{CO}_2$  from 7.0 to 100.1, the residual gas not estimated as  $\text{CO}_2$  or  $\text{O}_2$  (and assumed to be  $\text{N}_2$ ) varied over only narrow limits, the values for the five sampling periods being: 33.8, 31.7, 33.6, 31.3, and 31.0. In view of the fact that these represent five different lots of tissue sampled on five different days, the  $\text{N}_2$  values indicate not only the relative constancy of this fraction, but also furnish evidence that the method of extraction was satisfactory as to uniformity of behavior.

It is not suggested that the method employed in this experiment for determining the respiration of potato tubers would be a good one if the object was to measure the rate of respiration of potato tubers. In this test the amount of  $\text{O}_2$  available was constantly decreasing, and the concentration of  $\text{CO}_2$  was increasing, and usually it is not the rate of respiration under such conditions that is wanted, but rather the rate when the  $\text{O}_2$  concentration is about that of the atmosphere, and when the  $\text{CO}_2$  content is very low. But in this experiment the object was to obtain tissue with various proportions of internal gas in order to test the usefulness of methods of estimating the amount of gas in the tissue, and to note how the values so obtained could be applied in computation on respiration rate. The respiration method employed was satisfactory for such purposes.

#### SUMMARY

Apparatus is described for use in extracting by vacuum the gas from plant tissue, and the amounts found in such tissues as tubers of potato; fruits of squash, apple, and eggplant; roots of turnip and sweet potato; and corms of gladiolus, are given.

Procedures for estimating the carbon dioxide content of plant tissues by an aeration method are described.

Much more  $\text{CO}_2$  was found in the tissues by aeration than by the vacuum method. Usually not over one-half, and sometimes not more than one-tenth of the  $\text{CO}_2$  found by aeration could be removed from the tissue by evacuation.

The utility of these methods was tested in connection with the problem of measuring respiration under conditions in which the gas content of the tissue must be taken into consideration in obtaining an estimate of the amount of  $\text{CO}_2$  produced and the amount of  $\text{O}_2$  consumed. Potato tubers were stored in closed containers, and the change in the  $\text{CO}_2$  and  $\text{O}_2$  content not only of the air surrounding the tubers but also of the changes of these constituents in the tissues themselves were determined. Evidence was found that under such conditions the values for the  $\text{CO}_2$  content of the tissue by the aeration method are the ones that should be used in estimating the amount of  $\text{CO}_2$  produced, and not the  $\text{CO}_2$  values found by removing the gas from the tissue by vacuum.

With potato tubers a measurement of the  $\text{O}_2$  content of the tissue was not important in arriving at a satisfactory estimate of the amount of  $\text{O}_2$  consumed by respiration. In this tissue the  $\text{O}_2$  content is low and the proportion of internal  $\text{O}_2$  to the  $\text{O}_2$  in the air surrounding the tissue is so small that the correction to be made due to change in  $\text{O}_2$  in the tissue was found to be unimportant.

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# A TOXIC SUBSTANCE OCCURRING IN CERTAIN MAIZE COBS<sup>1</sup>

J. H. STANDEN<sup>2</sup>

## INTRODUCTION

In a study of *Nigrospora* dry rot of maize (*Zea mays* L.), the ears of an inbred, referred to as #113<sup>3</sup>, were found especially susceptible to infection. Because of this, meal prepared from cobs of this inbred was commonly employed in cultural studies of the pathogen. In the fall of 1939, six cobs of this inbred were ground, and a portion of the meal employed in making medium, by addition of water and agar agar. *Nigrospora oryzae* (B. & Br.) Petch refused to grow on the medium. On further study a substance toxic to the fungus, and to rats, was detected in the meal. Results of these studies were not published at the time because it was hoped that additional information could be obtained. The author has continued to search for this toxic material among cobs of inbred 113, and elsewhere since that time, but at this date has failed to detect it again. Because of the possible connection of this substance with the sporadic poisoning of live stock when farm animals are allowed to browse in cornfields after harvest (corn stalk disease), it seems advisable to publish the data obtained in these earlier studies.

## EXTRACTION AND DETECTION OF THE TOXIC SUBSTANCE FROM COBS

The toxic substance was removed from the dry cob meal by extraction with ether. A Soxhlet extractor with a wad of plugging cotton inserted in the bottom of the extraction chamber was found to be satisfactory for this purpose. After extraction of the meal for four hours, most of the ether was removed from the extract by evaporation. Water was then added to the residue and the mixture heated to remove the rest of the ether. A fatty substance separated from the water portion, and this was removed by filtration. In testing for the presence of the toxic substance in the extracted meal, the water fraction, and the fatty residue, aliquots equivalent to 2 grams of cob meal were made into 50-ml. portions of media with agar agar and 2 per cent dextrose Czapek's nutrient solution. Growth of *Nigrospora* occurred on the media containing cob meal and the fatty residue, but was entirely lacking on the medium containing the water extract. A test was made also of the effect of the substance on the growth of *Diplodia zeae*

<sup>1</sup> A considerable portion of this research was performed at Iowa State College, and has since been continued at Delaware Agricultural Experiment Station and Boyce Thompson Institute for Plant Research, Inc.

<sup>2</sup> The B. F. Goodrich Biochemical Laboratory at Boyce Thompson Institute for Plant Research, Inc., Yonkers 3, New York.

<sup>3</sup> One of a series of inbreds carried by Dr. C. S. Reddy, Botany and Plant Pathology Dept., Iowa State College, Ames, Iowa.

(Schw.) Lev. and *Gibberella saubinetii* (Mont.) Sacc. in culture. Growth of *Diplodia* was inhibited, but growth of *Gibberella* did not appear to be affected. A yield of 0.616 mg. of dry toxic material was extracted from a gram of the cob meal of inbred 113.

#### EXTENT OF OCCURRENCE AND SOME PROPERTIES OF THE TOXIC SUBSTANCE

In an attempt to procure the toxic substance from sources other than inbred 113, six cobs each of 16 other inbreds were ground, extracted, and the extract tested according to the method previously described. A trace of the toxic substance was detected in meal from two inbreds. The data indicated that the substance was not common in cobs of all inbreds. In another trial nine individual cobs of inbred L 289B were tested for the toxic substance, but it was detected in only one. This evidence indicated that the occurrence of this substance was not uniform in the cobs of any one inbred, but that it occurred in some cobs and not in others.

Tests were made to determine the general characteristics of the toxic substance. The water solution was colorless and had a very disagreeable taste. By evaporation a white powder was obtained that charred on heating and lost its toxicity. A trace of ash was left on ignition. Some of the toxic substance stored in the laboratory in the dry state for four months appeared to lose its toxicity. In three tests it was found that autoclaving solutions of the toxic substances at 16 pounds pressure for 4.5 hours did not change the ability of the substance to inhibit the growth of *Nigrospora* in cultures.

The growth of *Nigrospora* was inhibited when 1.232 mg. of the toxic substance were present in 50 ml. of nutrient agar. When half of this amount of the substance was used there was a little growth of the fungus. The toxic substance did not appear to be a by-product of the growth of *Nigrospora* because the fungus grew well on media containing meal made from *Nigrospora*-infected maize tissues.

#### EFFECT OF THE TOXIC SUBSTANCE ON RATS

Because of the markedly inhibiting effect of the toxic substance on growth of *Nigrospora oryzae* and *Diplodia zeae*, it seemed worth while to study the effect of this substance on laboratory animals. The rat was used as the test animal.<sup>4</sup>

The rats used in the test were either white or mixed, weight around 70 g., most of them young males, and none of them used in any previous experiment. The toxic substance, in aqueous solution, was administered to

<sup>4</sup> These experiments were performed with the kind assistance of Dr. B. H. Thomas of the Animal Chemistry and Nutrition subsection of the Iowa Agricultural Experiment Station, Ames, Iowa.

the etherized animals in two ways, namely intraperitoneally by means of a hypodermic syringe, and into the stomach through a very small rubber tube. Rats used as checks in the tests were given doses of an extract of cobs shown to contain no substance toxic to *Nigrospora*. These extracts were made up to the same strength, on the basis of weight of cob extracted, as the solution containing the toxic substance, and were administered to the check animals in equivalent amount in each case.

In a preliminary test two rats were given peritoneal injections of 1.232 and 0.318 mg. of the toxic substance. The rats died within two hours. Two other rats were given the same amounts of the substance by a stomach tube. The rats were alive after seven hours, but the one receiving the larger dose was dead at the end of twenty hours. The toxic solution was immediately tested with *Nigrospora* according to the method previously described and was found to have retained its inhibiting action on the fungus. The injected rats showed distress symptoms which included very evident discomfort, tendency for the eyelids to close, moping in the corner of the cage, shuddering, lowered head and difficulty in locomotion.

In a second test six rats were given injections of 0.20 to 1.6 mg. of the toxic substance. In this test all of the rats receiving the toxic substance died. None of the six rats receiving the check solution exhibited any ill effects. The data on the rats receiving the toxic substance are recorded in Table I.

TABLE I  
TIME INTERVAL BETWEEN PERITONEAL INJECTION OF TOXIC SUBSTANCE  
AND DEATH OF RATS

Dosage in milligrams	Minutes from injection until death of rat
0.2	57
0.4	54
0.6	38
0.8	34
1.2	29
1.6	3

All of the rats killed by the toxic substance in this test were subjected to post-mortem examination. No evidence of internal injury was detected in the rat receiving the largest dose. This rat died in 3 minutes, and it may be that gross injury symptoms had insufficient time to develop. The five remaining rats died within 29 to 57 minutes after injection. All of these showed the same internal symptoms, namely, general inflammation of the viscera, particularly the lower intestine. Pin-point lesions were so numerous on the inner wall of the lower intestine as to give it the appearance of red plush. Post-mortem examination of three rats from the check group failed to reveal these symptoms.



A test was made on the effect of prolonged autoclaving of a solution of toxic substance in relation to its toxicity to rats. The solution was autoclaved for 4.5 hours at 16 pounds pressure. A rat given 1.6 mg. of autoclaved solution of toxic substance by peritoneal injection died in less than 2 minutes. The test was repeated with smaller dosages using unautoclaved solution as a check. Two rats given injections of 1.2 and 0.6 mg. of the toxic substance in autoclaved solution died in 31 and 43 minutes respectively. Two rats given injections of 1.2 and 0.6 mg. of unautoclaved toxic substance died in 29 and 40 minutes respectively. The evidence indicated that autoclaving for 4.5 hours did not impair the toxicity of the aqueous solution of toxic substance.

Concurrently with the experiments of peritoneal injection, the toxic substance was administered orally by stomach tube to another group of rats. The rats were given one dosage per day for four days. A record was made of the daily weights of rats receiving the toxic substance and of check rats for a period of three weeks. The data are summarized in Table II. Rat number 3 of the group receiving doses of toxic substance died following administration of the dosage on May 2. Post-mortem examination showed inflammation of the viscera as in rats receiving peritoneal injections of the toxic substance.

TABLE II  
LETHAL AND DETRIMENTAL EFFECTS ON RATS OF THE TOXIC SUBSTANCE  
ADMINISTERED ORALLY

Date	Dosage in milligrams	Weight of rats in grams					
		Toxic substance			Check		
		Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3
April 30	0.2		89		77	83	79
May 1	0.2						
" 2	0.4	70	89	68	86	90	82
" 3	0.4	65	81	Died	88	96	84
" 4	*	62	75		96	104	Killed
" 6		67	76		99	113	
" 8		77	83		109	127	
" 16		110	104		128	153	
" 22		139	135		147	184	

\* Dosage discontinued after May 3.

All rats receiving toxic substance in this test exhibited the distress symptoms previously described, within 30 minutes. Loss of weight also occurred. A post-mortem examination of rat number 3 of the check group revealed none of the inflammation associated with the effect of the toxic substance. No distress symptoms or loss of weight occurred in the animals of this group. After administration of the toxic substance was discontinued, the rats showed a slow recovery, as indicated by the weight records.

## SUMMARY

A substance extracted from maize cobs was toxic to certain fungi and to rats. The substance was found in only a few of many cobs examined. It was a white, organic material, soluble in water, chloroform, and ether, and stable to autoclaving at 16 lbs. for 4.5 hours. When administered to rats by stomach or intraperitoneally, it caused multiple pin-point hemorrhages of the inner wall of the lower intestine and other characteristic symptoms. Very small dosages caused death. It is possible that this substance may be connected with the so-called "corn stalk" disease of horses and cattle.



# FURTHER STUDIES OF EMBRYOLESS SEEDS IN THE UMBELLIFERAE

FLORENCE FLEMION AND GLORIA UHLMANN

## INTRODUCTION

Some years ago a grower of dill sent for examination samples of fresh well-filled seeds from which he could obtain only 50 per cent germination. Studies revealed that most of the seeds which failed to germinate were without embryos (1). In these embryoless seeds the endosperm is present and from all appearances normal, while the small embryo which usually lies embedded in the endosperm at one end of the seed is lacking. Since the embryo represents such a small portion of the total weight of a given seed it has not been possible to separate the embryoless seeds by any method tried. Within a sample, the lack of embryos appears in all sizes of seeds.

Since seeds of so many of the commercially important members of the Umbelliferae are notoriously poor germinators, a survey was made as to the prevalence of embryoless seeds. In the nine species studied and reported below the occurrence of seeds with endosperm but without embryos was quite common. Ninety-nine per cent of the samples examined contained embryoless seeds in amounts which varied from 1 to 62 per cent. In many cases the percentage of embryoless seeds accounted for the lack of germination in what appeared to be well-filled good seeds. However, in some samples of several species, the percentage germination was still lower and this was due to the presence of immature embryos which frequently were incapable of germination. Embryoless seeds and immature embryos which fail to develop account for much of the low germination results obtained with fresh seeds of Umbelliferae.

## TECHNIQUE

Seeds of anise (*Pimpinella anisum* L.), caraway (*Carum carvi* L.), carrot (*Daucus carota* L.), celery (*Apium graveolens* L.), coriander (*Coriandrum sativum* L.), dill (*Anethum graveolens* L.), Florence or sweet fennel (*Foeniculum dulce* Mill.), parsley (*Petroselinum hortense* Hoff.), and parsnip (*Pastinaca sativa* L.) were examined. The seeds were obtained through the courtesy of Associated Seed Growers, Inc., W. Atlee Burpee Company, Ferry-Morse Seed Company, Pieters-Wheeler Seed Company, Waldo Rohnert Company, F. H. Woodruff and Sons, Inc., and Zeno Muggli, a seed grower. The lots represent various crops grown in different regions.

Duplicate lots of 50 or 100 seeds each were soaked in tap water for approximately 20 hours. By slitting the seeds longitudinally those without embryos could be readily detected. In embryoless seeds there is a cavity

TABLE I  
PER CENT OF EMBRYOLESS SEEDS AND GERMINATION OF  
VARIOUS LOTS OF CARROT SEEDS

Variety	Origin	Crop	Cutting test, per cent						Germination test	
			Embryoless	Empty	Deteriorated	Seeds with embryos			Date	Per cent*
						Total	Immature	Mature		
Chantenay	California	1941	11	0	0	89	—	—	February 1942	84†
	"	1944	21	0	4	75	5	70	November 1945	70
	"	"	28	0	2	70	0	70	"	63
	Colorado	"	18	0	1	81	1	80	October	79
	Idaho	"	3	0	1	96	1	95	November	94
	"	"	5	0	0	95	2	93	"	93
	"	"	10	0	0	90	1	80	"	85
	"	"	10	1	2	86	3	83	December	80
	"	"	11	0	4	85	11	74	November	76
	California	1945	24	0	7	69	4	65	December	62
Chantenay, Improved	New Mexico	"	7	0	1	92	0	92	"	84
	"	"	17	0	1	82	1	81	"	82
	Idaho	1941	10	0	1	89	—	—	March 1942	91
	"	"	25	3	0	72	—	—	February	67
	California	1945	18	0	5	77	6	71	November 1945	71†
	"	"	23	0	6	71	2	69	"	68
	"	"	28	9	2	61	1	60	"	64
	"	"	31	6	2	61	1	60	"	66
	California	1941	13	0	0	87	—	—	March 1942	84†
	Idaho	"	13	0	0	87	—	—	February	78†
Chantenay, Red Core	Ohio	1941	7	0	0	93	—	—	March 1942	89
Danvers	California	1945	22	0	0	78	2	76	December 1945	63
	"	"	24	4	3	69	2	67	October	56
Goldinhardt	California	1942	25	0	4	71	—	—	January 1945	60
Hutchinson	California	1941	14	0	0	86	—	—	February 1942	71†
	"	1945	37	0	1	62	8	54	November 1945	68†
Imperator	California	1944	20	0	4	76	17	59	November 1945	58
	Colorado	"	9	0	2	89	10	79	"	77
	Idaho	"	3	0	0	97	0	97	"	90†
	"	"	10	0	0	90	0	90	"	93
	"	"	13	0	0	87	3	84	"	81
	"	"	14	4	4	78	4	74	"	75
	"	"	17	1	1	81	2	79	"	71
	California	1945	7	0	5	88	4	84	"	87
	"	"	8	0	2	90	3	87	December	68
	"	"	18	0	0	82	2	80	November	68
	"	"	18	4	2	76	3	73	December	72
	"	"	18	0	0	82	0	82	November	74
	"	"	21	10	6	63	3	60	December	67
	"	"	24	7	5	64	5	59	"	63
Imperator, Improved	California	1945	13	0	2	85	0	85	December 1945	85†
	"	"	15	0	0	85	4	81	"	86†

TABLE I—(Continued)

Variety	Origin	Crop	Cutting test, per cent						Germination test	
			Em- bryo- less	Empty	De- terio- rated	Seeds with embryos			Date	Per cent*
						Total	Imma- ture	Ma- ture		
Imperator, Long	California	1945	9	0	1	90	0	90	December 1945	89†
	"	"	14	0	3	83	2	81	November "	75
	"	"	14	2	2	82	2	80	December "	90
	"	"	16	0	2	82	1	81	" "	84
	"	"	20	0	2	78	6	72	November "	71†
	"	"	20	3	1	76	2	74	" "	77†
Nantes	California	1941	22	0	0	78	—	—	March 1942	72†
	"	"	31	0	0	69	—	—	February "	65†
	Idaho	1944	14	0	3	83	7	76	October 1945	74†
	"	"	15	0	1	84	0	84	" "	66†
No. 122	California	1945	9	0	0	91	2	89	October 1945	88
St. Valery	California	1945	17	0	4	79	0	79	October 1945	72

\* Laboratory test except where otherwise indicated.

† Greenhouse soil test.

in the endosperm at one end of the seed where the embryo would normally be found (1, Fig. 1 C). Some seeds were entirely empty containing neither endosperm nor embryo while in others that were non-viable both the endosperm and embryo had deteriorated.

At approximately the time of the cutting tests the percentage germination was determined. Seeds were tested in the laboratory on moist filter paper in Petri dishes at 20° to 30° C. alternated daily (8 hr. at 20° C. and 16 hr. at 30° C.) as well as in soil plantings in a greenhouse maintained at approximately 21° C. (70° F.) Duplicate lots of 100 seeds each were used in each test. The percentage germination recorded in the tables is usually that obtained in the laboratory, but when the germination was greater in the soil this value was used and is so indicated in the tables.

## RESULTS

Approximately one-fourth of the 200 samples of seeds examined was carrot. The data in Table I were obtained from 54 lots grown principally in California and Idaho from 1941 to 1945. The percentage of seeds without embryos varied from 3 to 37 per cent with an average of 16 per cent. Besides embryoless seeds approximately three-fourths of the lots examined contained from 1 to 17 per cent immature embryos. Thus, another factor can and does lower germination since these immature embryos may or may not germinate. When the percentage germination is compared with the

**TABLE II**  
PER CENT OF EMBRYOLESS SEEDS AND GERMINATION OF VARIOUS LOTS OF CELERY SEEDS

Variety	Origin	Crop	Cutting test, per cent						Germination test	
			Embryoless	Empty	Deteriorated	Seeds with embryos			Date	Per cent*
						Total	Immature	Mature		
Easy Blanching	Unknown	?	11	0	1	88	2	86	July 1945	78
	California	1943	3	0	4	93	4	89	September " 1945	87
	"	"	11	0	2	87	11	76	July " "	74
	"	1944	9	0	0	91	3	88	September " "	84†
	"	"	11	0	2	87	15	72	October " "	69†
Emperor	California?	1941	8	0	0	92	—	—	February 1942	85†
	California	1943	2	0	1	97	8	89	July " 1945	87
	"	1944	13	0	1	86	7	79	" " "	77†
Fordhook	California	1942	8	0	2	90	6	84	July " 1945	90
	"	1943	8	0	3	89	4	85	" " "	89
	"	1944	3	0	1	96	1	95	" " "	96
Giant Pascal	California	1941	3	1	0	96	—	—	March 1942	93
	"	1944	4	0	0	96	8	88	July " 1945	80
	"	"	8	0	2	90	19	71	" " "	72
	"	"	14	0	1	85	4	81	August " "	82†
	"	1945	5	0	3	92	11	81	November " "	83†
Golden Detroit	California?	1941	6	0	0	94	—	—	March 1942	60
Golden Plume	Unknown	?	6	0	1	93	8	85	July 1945	80
	California	1942	17	0	2	81	23	58	September " "	5
	"	1944	3	0	2	95	12	83	August " "	81†
	"	"	9	0	1	90	48	42	July " "	71†
	"	"	20	0	2	78	31	47	October " "	54
Golden Self Blanching	California	1943	7	0	10	83	25	58	July " 1945	64
	"	"	9	0	4	87	31	56	" " "	60
	"	"	13	0	2	85	9	76	September " "	71
	"	1944	6	0	4	90	38	52	July " "	67
Green Florida Pascal	California	1943	7	0	4	89	4	85	July 1945	87
Kilgore's Pride	California	1944	7	0	12	81	44**	37	July 1945	52
Short Top Pride	California	1944	9	0	6	85	36**	49	July " 1945	65
	"	"	5	0	13	82	25	57	" " "	59
Utah	California	1944	2	0	2	96	5	91	July " 1945	86
	"	"	10	0	2	88	9	79	" " "	74
Utah or Golden Crisp	Unknown	1943	14	0	5	81	7	74	September " 1945	76
	California	"	6	0	4	90	2	88	" " "	86
	"	1944	5	0	4	91	3	88	August " "	85
Utah Pascal	California	1944	4	0	3	93	3	90	July 1945	89
White Plume	California?	1941	14	3	0	83	—	—	March 1942	74
	California	1943	20	0	3	77	17	60	August " 1945	55
	"	1944	6	0	3	91	26	65	September " "	80
	"	"	8	0	3	89	1	88	" " "	90

\* Laboratory test except where otherwise indicated.

† Greenhouse soil test.

\*\* Part of endosperm gelatinous.

per cent of mature, immature, and total number of seeds with embryos, it is seen that in some cases the total germination approximates the number of mature embryos while at other times it approaches the number of filled seeds.

In regard to differences in variety there were usually not adequate numbers of samples for comparison. However, in two varieties of carrot, the 12 lots of Chantenay and the 14 lots of Imperator had approximately the same degree of embryoless seeds.

Forty samples of celery seeds representing 14 varieties grown principally in California were examined. The percentage of embryoless seeds varied from 2 to 20 per cent (Table II). In celery all of the samples examined contained immature embryos and in amounts from 1 to 48 per cent. In about half of the lots some of the immature seeds germinated and in these cases the percentage germination was intermediate between the percentage of mature seeds and the number of filled seeds. It is interesting to note that in celery the percentage of seeds lacking embryos is not as great as in carrot but the frequency and number of immature embryos is considerably greater (Table VI). Within a given species, however, no correlation was observed between the percentage of embryoless seeds and the percentage of immature embryos.

The data obtained with eight varieties of parsley seeds are presented in Table III. With but one exception these were produced in California. Parsley seeds were found to have about the same variation in percentage of seeds without embryos as carrot. Approximately one-fourth of the samples were examined for immature embryos and in these the average was 4 per cent (Table VI). Thus, in parsley as in celery and carrot, immaturity of the embryo together with embryoless seeds affect percentage germination.

As published earlier (1), dill seeds may be embryoless up to about 60 per cent. The 12 lots described in the previous publication are incorporated (designated by \*\*) in Table V plus an additional 27 lots studied. There is considerable variation in the percentage of embryoless seeds found in this species and in the 39 lots considered here from 4 to 62 per cent of the seeds lack embryos.

Embryoless seeds were also found in anise, caraway, and coriander (Table IV). Fennel seeds, as in the case of dill, also vary considerably in the degree of frequency of embryolessness (Table IV). The maximum amount of seeds lacking embryos was 62 per cent for dill and 58 per cent for fennel, while the averages were 24 and 34 per cent, respectively. In most cases the percentage germination of fresh seeds closely approximated the per cent of seeds with embryos. Immaturity of embryos was not particularly noted in these samples.

Embryoless seeds as shown in the tables were found in all of the 54 lots of carrot, 40 lots of celery, 39 lots of dill, 12 lots of fennel, and 31 lots of



TABLE III  
PER CENT OF EMBRYOLESS SEEDS AND GERMINATION OF VARIOUS LOTS OF PARSLEY SEEDS

Variety	Origin	Crop	Cutting test, per cent						Germination test	
			Em- bryo- less	Empty	De- terio- rated	Seeds with embryos			Date	Per cent*
						Total	Imma- ture	Ma- ture		
Curled	Probably foreign	1940	4	0	0	96	—	—	March 1942	92
	California	"	11	0	0	89	—	—	" "	80†
	"	1941	16	0	0	84	—	—	" "	80
	"	"	17	0	0	83	—	—	" "	78†
	"	"	23	0	0	77	—	—	" "	71†
	"	"	27	0	0	73	—	—	" "	69†
	"	"	28	0	0	72	—	—	" "	73†
Curled, Moss	California	1941	27	4	0	69	—	—	February 1942	67†
	"	1943	14	0	0	86	2	84	October 1945	84
	"	1944	14	0	0	86	3	83	November "	89
	"	"	19	0	0	81	3	78	" "	73†
	"	"	29	4	0	67	4	63	October "	40†
Curled, Spe- cial Moss	California	1941	29	0	4	67	—	—	March 1942	72
Curled, Ex- tra Triple	California	1941	18	0	0	82	—	—	February 1942	78
Emerald or Extra Dwarf Curled	California	1942	23	0	4	73	—	—	January 1945	70
Hamburg or Rooted	California	1940	24	0	0	76	—	—	March 1942	71
	"	"	29	0	0	71	—	—	" "	71
	"	1941	22	0	0	78	—	—	October 1945	71
	"	1943	22	1	0	77	5	72	" "	68†
	"	1944	11	3	0	86	4	82	" "	76†
Paramount	California	1941	13	0	0	87	—	—	February 1942	68
	"	"	18	0	0	82	—	—	" "	78
	"	"	33	0	17	50	—	—	March "	45†
Plain- leaved	California	1939	2	0	0	98	—	—	March 1942	94
	"	1941	26	0	0	74	—	—	" "	74
	"	"	36	0	0	64	—	—	" "	58
	"	1943	6	2	0	92	4	88	October 1945	82†
	"	1944	33	0	0	67	3	64	November "	73
Unknown	California	1940†	21	0	0	79	—	—	February 1941	73
	"	1941	12	0	0	88	—	—	" 1942	80†
	"	"	17	1	0	82	—	—	" "	75

\* Laboratory test except where otherwise indicated.

† Greenhouse soil test.

‡ See (1, p. 158-159).

TABLE IV  
PER CENT OF EMBRYOLESS SEEDS AND GERMINATION OF VARIOUS LOTS OF ANISE, CARAWAY,  
CORIANDER, FENNEL, AND PARSNIP SEEDS

Species		Origin	Crop	Cutting test, per cent				Germination test	
				Em- bryo- less	Empty	De- terio- rated	Seeds with em- bryos	Date	Per cent*
Anise		Unknown	?	6	0	0	94	March 1942	88
Caraway		Unknown	?	0	12	15	73	November 1941	71
		"	1937	9	0	0	91**	March 1942	0
		"	1939	25	0	0	75**	"	0
		Holland California	1940 1943	8 12	0 0	0 4	92 84	" January 1945	27 67
Coriander		Holland Morocco	1940 "	3 3	5 3	25†† 0	67 94	February 1942 March "	35† 92†
		Unknown	1941	1	0	1	98	January 1945	89†
Fennel, Florence or Sweet		California	?	51	0	4	45	February 1942	39†
		"	?	54	0	1	45	March "	40
		"	?	58	0	2	40	" "	38†
		Holland	1938	18	3	5	74	February "	49
		Unknown	1939	9	0	0	91	" "	76
		California	1941	35	0	8	57	" "	61
		"	"	44	0	2	54	" "	48
		"	"	48	0	1	51	" "	56
		New Jersey	1942	11	3	1	85	November 1945	23
		California	1944	6	0	2	92	" "	76
		"	1944†	43	2	33	22	" "	4
		"	1945	26	2	4	68	" "	62†
Parsnip	All American	Idaho	1941	19	7	1	73	March 1942	54
		Oregon	1944	2	0	1	97	October 1945	100†
	Guerney	California	1943	5	2	6	87	January 1945	54
	Harris Model	Oregon	1941	3	3	0	94	March 1942	97†
		"	"	5	0	1	94	" "	91†
	Hollow Crown	California	1941	13	0	0	87	February 1942	82†
		California & Idaho mixed	"	12	0	0	88	" "	79†
		Oregon	"	2	0	0	98	March "	95†
		"	1943	10	1	1	88	October 1945	49†
	Unknown	California	1941	12	1	0	87	February 1942	77
		Connecticut	"	9	3	0	88	" "	78†
		New Jersey	"	19	4	0	77	" "	56
		Oregon	"	2	1	0	97	" "	98†
		Pennsylvania	"	7	40	0	53	" "	41†
		North Dakota	1942	0	19	9	72	October "	59

\* Laboratory test except where otherwise indicated.

\*\* Contents of many discolored.

† Greenhouse soil test.

†† Seeds with insect holes.

‡ Seeds injured by adverse weather conditions during harvest.

parsley that were examined. Only one of the five lots of caraway and one of the 15 lots of parsnip contained no evidence of embryoless seeds. The high-

TABLE V

PER CENT OF EMBRYOLESS SEEDS AND GERMINATION OF VARIOUS LOTS OF DILL SEEDS

Origin	Crop	Cutting test, per cent				Germination test	
		Embryoless	Empty	Deteriorated	Seeds with embryos	Date	Per cent*
Unknown	? **	60	0	5	35	February 1941	24
"	1939	7	2	0	91	March 1942	92
"	"	25	4	1	70	November 1941	55
Connecticut	" **	39†	2	0	59†	March " "	54††
Denmark	"	17	0	0	83	" 1942	79†
Holland	"	4	1	0	95	" " "	92†
"	" **	8	1	0	91	" 1941	88
"	" **	14	0	1	85	" " "	82
Holland or Denmark	" **	12	0	0	88	April " "	88
American	1940 **	62	1	0	37	March 1941	29
California	" **	9	0	4	87	" " "	87
"	" **	13	0	0	87	" " "	87
"	" **	48	0	0	52	January " "	46
New York	" **	9†	0	0	91†	March " "	94††
North Africa	" **	11	0	0	89	April " "	83
North Dakota	" **	7	0	0	93	March " "	87
"	"	13	0	0	87	November " "	86†
California	1941	25	0	0	75	March 1942	74†
"	"	27	5	0	68	December 1941	63†
"	"	31	4	0	65	February 1942	57†
"	"	38	2	0	60	" " "	49†
"	"	45	4	1	50	March " "	50†
Colorado	"	19	0	0	81	" " "	64†
Idaho	"	28	0	3	69	" " "	73
"	"	43	1	15	41	" " "	30
North Dakota	"	25	0	0	75	" " "	79
"	"	34	0	0	66	" " "	58
Oregon	"	8	3	0	89	" " "	93†
"	"	10	0	0	90	February " "	87†
"	"	18	1	1	80	October 1945	64
"	"	24	0	0	76	February 1942	57†
"	"	25	3	0	72	" " "	52
Unknown	"	31	0	1	68	" " "	61†
North Dakota	1942	5	2	5	88	November 1942	56
Oregon	"	18	0	1	81	October 1945	73
California	1943	26	8	0	66	January " "	72
"	"	43	0	0	57	October " "	58†
Oregon	1944	31	0	1	68	" " "	65
"	1945	9	2	1	88	" " "	82

\* Laboratory test except where otherwise indicated.

\*\* See (1, p. 159, Table I).

† Greenhouse soil test.

†† Duplicates of 50 seeds each.

‡ Only 1 set of 100 seeds.

est percentages noted were in dill and fennel with 58 and 62 per cent respectively, while celery and parsnip did not exceed 20 per cent. Parsley and carrot were intermediate with 36 and 37 per cent. The averages for these species are in the same order as the maximum values (Table VI).

TABLE VI  
SUMMARY OF EXTENT OF EMBRYOLESSNESS AND EMBRYO IMMATUREITY FOUND  
IN NINE SPECIES OF UMBELLIFERAE

Species	Embryolessness				Immature embryos			
	Number of lots examined	Per cent without embryos			Number of lots examined	Per cent		
		Average	Maximum	Minimum		Average	Maximum	Minimum
Carrot	54	16	37	3	44	4	17	0
Celery	40	8	20	2	36	14	48	1
Dill	39	24	62	4	0	—	—	—
Parsley	31	20	36	2	8	4	5	2
Parsnip	15	8	19	0	0	—	—	—
Fennel	12	34	58	6	0	—	—	—
Caraway	5	11	25	0	0	—	—	—
Coriander	3	2	3	1	0	—	—	—
Anise	1	6	—	—	0	—	—	—
For all species	200	17	62	0	88	8	48	0

Within the limits of the experiments, no significant differences were apparent between embryoless seeds, variety, or crop year in any species studied.

#### DISCUSSION

The approximate average of embryoless seeds found in celery and parsnip was 8 per cent, carrot 16 per cent, parsley 20 per cent, dill 24 per cent, and fennel 34 per cent. Thus, as far as plant production is concerned approximately one-twelfth of the celery seed crops and one-third of the fennel seed crops are worthless, due to this condition. In addition, a considerable number of the seeds with embryos in some species are also worthless, due to immaturity of the embryos. However, if these savory seeds are to be used for culinary purposes or essential oils, they are of value, since the aromatic principles are found in the fruits and coats and not in the embryos.

Carrot seeds are not used as aromatics. Many crops yield poorly germinating seeds and the minimum standard Federal germination requirement

is 55 per cent. The average yearly production of carrot seed in the United States from 1939 to 1943 was approximately 1,755,000 pounds (4). From the studies above it is estimated that about one-sixth of the crops is worthless, due to embryoless seeds. This is costly in terms of production, handling, storage, and subsequent seedling production. Furthermore, an average of approximately 3 per cent of the filled seeds contained immature embryos, thus causing an additional hazard as far as germination quality and seedling vigor are concerned.

It is interesting to note that in celery the amount of embryoless seeds is not as great as in carrot, but the number of immature embryos is considerably higher. About one-twelfth of the celery seeds lack embryos and an additional one-seventh contains immature embryos which frequently do not germinate.

Much of the commercial seed production of the Umbelliferae in this country occurs in the western states. Watson (5) in discussing the culture of caraway and celery seed points out the differences in the European and American methods of harvesting. In Europe much of the material is hand picked. In the data presented above only 5.5 per cent of the lots studied were of foreign origin. Most of the lots of parsley (97 per cent) and of celery (87 per cent) were grown in California, while in the case of carrot 63 per cent of the lots was produced in California and 28 per cent in Idaho. With dill, only about one-fourth of the samples examined came from California. Regardless of the source these seed lots contained embryoless seeds.

A review of the literature was presented in the earlier publication on dill (1). The articles by Borthwick were cited (1) in regard to the germination failure of a considerable percentage of carrot seeds due to defective or immature embryos and he stated that Cole, in unpublished work, had found that some do eventually germinate. Since some of these underdeveloped embryos germinated when kept in a germinator for several months (instead of within a few weeks as for mature seeds), it appeared to Borthwick that an important cause of low germination in carrots was perhaps due to dormancy in these immature embryos. Munn and Heit (3) reported the presence of embryoless seeds in some of the Umbelliferae, especially dill and fennel, but they did not present any data. They warned seed analysts to use only the total percentage as the actual measure of germination capacity. Heit (2) pointed out that some dealers and growers falsely believe these worthless embryoless seeds to be viable and classify them as "live" or "hard" seeds. He was unable to remove by recleaning the embryoless carrot seeds which appeared in all sizes of seed. In one lot of dill where he obtained only 42 per cent germination, he found upon examination 49 per cent to be embryoless. Wright (6) reported that in two lots of carrot 39.5 per cent and 87 per cent of the seeds were embryo-

less. To date, no reference in the literature has been found regarding embryoless seeds in celery, parsley, or parsnip.

Embryoless seeds were found in all of the nine species of the Umbelliferae studied in the survey presented above. In no other family has such a large percentage of embryoless seeds been reported. Usually, the recorded frequency in other groups has been less than one per cent.

It would be of tremendous value if these embryoless seeds could be eliminated either by a separation method or better still by control of the contributing factor or factors. If the cause is of an hereditary nature perhaps it could be overcome genetically. Some have suggested it might be due to an irregularity during or following fertilization which could or could not be influenced by various external conditions. Various non-hereditary factors are being studied in an attempt to find some solution to this problem.

#### SUMMARY

An examination of two hundred different lots of seeds of anise, caraway, carrot, celery, coriander, dill, fennel, parsley, and parsnip revealed that the occurrence of seeds without embryos but with apparently normal endosperm is common and may reach a frequency of 50 per cent or more. The occurrence of embryoless seeds and of immature embryos in some species accounts for the great variation in the germinative capacity of fresh seeds.

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# FACTORS INFLUENCING VITAMIN C CONTENT OF ASPARAGUS, BANANA, AND SEEDLINGS OF GARDEN PEA DURING GROWTH OR IN STORAGE<sup>1</sup>

NORWOOD C. THORNTON

The vitamin content of fruits and vegetables is affected materially by the conditions of growth and the methods of handling the harvested product. Thus, it is essential that information be gathered regarding the factors influencing the natural production or loss of vitamin C in plant tissue. It is, the purpose of this paper to show the effect of some of these factors, namely, light, darkness, and ethylene during growth, and the presence of oxygen, carbon dioxide, and ethylene during storage, on the vitamin C content of asparagus, banana, and garden pea.

## MATERIAL AND METHODS

Asparagus (*Asparagus officinalis* L.) plants were growing in a seven-year-old bed in the Institute gardens. Bananas (*Musa sapientum* L. var. Gros Michel) were obtained from United Fruit<sup>2</sup> steamers in New York City. Garden pea (*Pisum sativum* L. var. Alaska) seedlings were grown in greenhouse flats in a mixture of peat moss and sand in a dark room or in the greenhouse for a period of six days from planting when the seedlings were approximately 4 cm. high before being exposed to ethylene. Sampling of these tissues for storage or analyses of vitamin C content was done at various stages of maturity as indicated in the discussion of experimental results.

The effect of sunlight and darkness on growing asparagus was studied in the garden where the shoots were allowed to grow either in the light or were sampled below ground, or mounds of earth were built up over the asparagus crown or a portion of the bed was covered with three thicknesses of black cloth. The effect of various gases on the plant tissue was studied in the laboratory by storing the tissue in sealed tin cans or in air-tight glass cases. The gases used (oxygen, carbon dioxide, and ethylene) were obtained from tanks of the compressed gases.

The asparagus and pea tissues were sampled by cutting off the bud or portion of stem tissue and then quickly weighing and placing the tissue under the acids used for extraction. Bananas were sampled by cutting out a portion of the finger, then quickly peeling, weighing, and covering with

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<sup>2</sup> The writer is indebted to Mr. G. L. Poland of the United Fruit Company Research Laboratory for furnishing the bananas used in these experiments.

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acids. Whenever duplicate samples were taken the exposed cut surface that had stood during the weighing operation was removed by making a fresh cut just previous to weighing the second portion of the tissue. By this procedure, triplicate (14) samples that checked consistently have been obtained from the same finger. The fingers from any one hand are quite uniform in vitamin C content, varying from 12.7 to 13.3 mg. per 100 g. across the hand and from 0.0 to 0.3 mg. for inside and outside paired fingers. Selections of fruit of one color gave in general a fairly uniform vitamin C content, especially suitable for the study of effect of gases on the vitamin C content during ripening.

A weighed quantity of tissue was placed in a mortar, covered with the extracting mixture of acids, and ground with a pestle with the aid of acid washed quartz sand. The thoroughly ground tissue was transferred to a 200 cc. volumetric flask, made up to volume with the acid mixture, thoroughly mixed, centrifuged, and aliquots used for titration with a solution of 2,6-dichlorophenolindophenol. The end point was reached at the point where the pink color of the dye persisted for approximately 10 seconds with constant agitation of the solution. Potentiometric determination of ascorbic acid in the tissue extract, using the mercury-platinum electrode devised by Harris and co-workers (6), agreed with visual titration used in this report. The dye solution was standardized daily by titration of pure ascorbic acid dissolved in the mixture of acids used for extracting the tissue. The strength of ascorbic acid was determined by the method of Ballentine (1).

The acid extracting medium used was equal parts of M/2 sulphuric and M/4 metaphosphoric acid (15). This medium prevented oxidation of ascorbic acid to dehydroascorbic acid during extraction of the tissue, thus eliminating the necessity for reducing the extract with hydrogen sulphide which Harris and Poland (7) found necessary with bananas when acetic acid was used. Because of many diversified reports on methods to eliminate strong acids, especially sulphuric acid, from extracting media many tests were made to compare the efficiency of various proposed methods on extracting vitamin C from bananas. Sulphuric acid medium has been condemned by many investigators since it was first used by Tillmans *et al.* (16, 17) and then modified as at present used by Mack and Tressler (8), and reported upon favorably by Gould, Tressler, and King (3), in order to obtain comparable results between the bioassay and the chemical method for the determination of ascorbic acid. Granted that sulphuric acid causes fading of the dye when the acid is run into the dye, so do most other extractants if used in sufficient strength to inhibit oxidation of ascorbic acid in bananas during the extraction procedure. However, where the procedure is reversed (dye into acid medium) the situation is entirely different when testing fresh biological material. Possibly not sufficient at-

tention has been paid to the statement by Mindlin and Butler (9, p. 674): "In such strongly acid media as are used in the usual titration procedure, the rate of fading of the dye is enough to cause an appreciable error at the concentrations of oxidized dye prescribed by our method (photoelectric colorimetric determination of ascorbic acid). In the titration procedure, in which complete reduction of the dye is almost instantaneous until the end-point is reached, this error of fading is minimized." Comparison of the Bessey (2) and Morell (10) methods with the sulphuric acid method used in this paper was made on the same finger of different lots of bananas with very interesting results. The Morell method is of no value in this case because at pH 3.5 the ascorbic acid is oxidized, part of which can be recovered by reduction with hydrogen sulphide. The Bessey method and sulphuric acid extractant gave identical results either upon visual titration or when determined with the photoelectric colorimeter. Also in these determinations the results as determined by titration and by photometric method were in agreement. Where the titration procedure was used the end-point was obtained quickly and was stable over a period of one minute and this compared with a 15-second reading on the photometric colorimeter; longer reading in the latter case of course gave a slow drift as the excess of dye was decolorized. Hydrogen sulphide reduction of these solutions was found unnecessary.

#### EXPERIMENTAL RESULTS

*Asparagus.* The increase in ascorbic acid content may be ten-fold from the dormant bud 15 cm. underground to the actively growing bud on a side branch 100 cm. above the soil. Wolf (18, 19) has reported green asparagus

TABLE I  
ASCORBIC ACID CONTENT OF ASPARAGUS BUDS TAKEN FROM PLANTS AT  
VARIOUS STAGES OF GROWTH

Position of bud	Height in cm. below (-) or above (+) ground	Mg. ascorbic acid per 100 g. fresh weight
Dormant at crown	(-) 15.3	15
Enlarging at crown	(-) 15.3	22-30
Growing	(-) 5.1 to 10.4	23-34
Above ground	(+) 1.3 to 10.2	70-95
Above ground	(+) 10.3 to 100.2	95-136
Side branch of main shoot	(+) 89.3 to 100.2	150

tips to contain twice as much vitamin C as the bleached or white ones. As shown by the data in Table I, this increase in vitamin C occurs gradually with the growth of the tissue above the ground. However, there is a marked difference in vitamin C content of the bud underground, or in darkness, and the bud just above ground, or in light. This marked increase

from 23 to 34 mg. of vitamin C per 100 g. in the bud underground to 79 to 95 mg. per 100 g. in the bud above ground usually would be attributed to the action of sunlight in the manufacture of food reserves, which has been established by Reid (11) and Hamner and Parks (5) for other plants. It is at this stage of growth (10 to 15 cm. above ground) that the asparagus shoot is cut for food. If the shoots were left to grow to a greater height until the side branches opened from the main bud, there would be a much higher vitamin C content but the asparagus would not be in demand as a food.

The vitamin C content of the stem tissue was always lower than the vitamin C content of the bud tissue. A section of stem tissue 5 cm. below the bud usually contained from 50 to 60 per cent, and a section of tissue 15 cm. below the bud contained about 30 per cent as much vitamin C as the bud tissue. This distribution of vitamin C in the asparagus plant was found to be fairly uniform regardless of height of the plant.

The asparagus roots growing from the crown contained about the same vitamin C content as the dormant buds, 15 mg. per 100 grams of tissue.

The increase in vitamin C content as the asparagus shoot emerged from the soil was attributed to the effect of light. However, this factor was tested and as the data in Table II show, light was not entirely responsible for the rapid increase in vitamin C content of the tissue, since the tissue in-

TABLE II  
ASCORBIC ACID CONTENT OF ASPARAGUS TISSUE GROWING IN SUNLIGHT  
AND UNDER BLACK CLOTH

Tissue taken for analyses	Mg. ascorbic acid per 100 g. of tissue											
	Height above ground; tissue growing in sunlight or darkness											
	3.8 cm.				6.4 cm.				12.1 cm.			
	Light		Dark		Light		Dark		Light		Dark	
	Wet wt.	Dry wt.	Wet wt.	Dry wt.	Wet wt.	Dry wt.	Wet wt.	Dry wt.	Wet wt.	Dry wt.	Wet wt.	Dry wt.
Bud (2 cm.)	97	856	80	819	94	829	72	734	92	807	75	766
Stalk below bud (5.1 cm.)	39	536	39	585	50	683	28	414	59	802	33	500
Next 5.1 cm. of stalk									32	294	24	215

creased in vitamin C even when emerging from the soil in darkness. Although the values are usually lower for tissue growing in the dark than in light, and continue to become lower as growth continues, the differences for the bud tissue are not as great as expected. A further test of the light factor was made by building up a mound of loose soil over the asparagus crown so that the shoot would have to grow through an additional 25 cm.

of soil before emerging into the light. The tissue was analyzed when still 10 cm. under the soil (having grown 15 cm. above the surface of the soil surrounding the check plants) with results that were not significantly different from the control lots of the same height grown in light, regardless of whether the ascorbic acid was calculated on the fresh or dry weight basis. Since it was not possible for light to have penetrated to the asparagus shoot it must be concluded that some factor other than light is responsible for the change in vitamin C content of the asparagus shoot as it increased in length.

Asparagus shoots 20 cm. above ground were harvested and brought into the laboratory to be stored for various periods in air and in various concentrations of oxygen and mixtures of oxygen and carbon dioxide at 10° and 20° C. The results show that the bud tissue held in air lost very little ascorbic acid during the first five hours of the storage period immediately after harvest, but when the tissue was held longer the rate of loss became much greater. Storage in various concentrations of oxygen had no definite effect upon the rate of loss of vitamin C as compared with the control. However, as previous results (13) have repeatedly shown, the accumulation of as little as 5 per cent carbon dioxide in the storage atmosphere caused a decidedly more rapid loss of vitamin C and the extent of the loss increased with increase in concentration of carbon dioxide. Removal of the asparagus tissue from the carbon dioxide and ventilation with air or increased concentrations of oxygen caused no recovery or retardation in the loss of vitamin C content of the tissue. Likewise all tests made to determine the presence of dehydroascorbic acid in the extracts from these tissues demonstrated the completeness of the loss of ascorbic acid as a result of the various treatments.

*Banana.* Green bananas contain approximately 15 mg. of ascorbic acid per 100 g. of tissue and, as ripening progresses, the vitamin C content slowly decreases as indicated by the data in Table III and Figure 1. However, the banana contains from 10 to 12 mg. of vitamin C per 100 g. of tissue at the desirable eating stage, depending upon whether one selects a soft 50 per cent brown-peel fruit or a firm yellow-peel fruit. After the peel becomes brown and the pulp develops a mushy consistency and a fermented aroma, the vitamin C content decreases to approximately 8 mg. Changes in temperature of storage, increases in oxygen concentration of the atmosphere, and the presence of added ethylene (1-500 to 1-8000 parts of air) in the storage room have only the effect of altering the rate of ripening of the fruit without altering the vitamin C content other than that usually found to occur with ripening. In some tests fruit treated with ethylene, then allowed to ripen in normal air, contained slightly more vitamin C than fruit ripening without the benefit of added ethylene.

The presence of carbon dioxide in the storage atmosphere has a detri-

mental effect upon the vitamin C content of ripening bananas as shown by the data in Table III. Apparently a small amount of carbon dioxide produced through the respiration of the fruit had some detrimental effect and this gave an indication as to what could be expected if the fruit was held for long periods in closed storage. When 8 or 16 per cent of carbon dioxide was placed in the storage atmosphere, there was considerable reduction in the vitamin C content, and treatment of the extract with  $H_2S$

TABLE III  
EFFECT OF VARIOUS PERCENTAGES OF CARBON DIOXIDE ON THE ASCORBIC ACID  
CONTENT OF BANANAS DURING RIPENING AT 19° C.

Color of fruit	Color index No.	Mg. ascorbic acid per 100 g. tissue			
		% Carbon dioxide in storage atmosphere			
		0	3-5	'8	16
Dark green	1	15.5	—	—	—
Light green	2	16.0	14.0	11.0	10.0
Yellowish green	3	14.9	12.0	8.0	6.0
Greenish yellow	4	13.8	—	—	—
Yellow green tip	5	12.5	11.7	7.2	5.0
Full yellow	6	11.8	—	—	—
Yellow brown fleck	7	10.1	9.8	5.8	3.8
50% Brown, pulp soft	8	10.1	9.0	7.8	5.4
Brown pulp, very soft	9*	9.9	—	—	—

\* Because fruit was not considered a marketable banana, it was not photographed.

showed the absence of partially oxidized ascorbic acid. This effect of the gas on the vitamin C content of the fruit was more pronounced with continuous treatment of green fruit. Fruit allowed to ripen previous to treatment showed very little reduction in vitamin C content even with as much as 60 per cent of carbon dioxide.

In some tests the carbon dioxide was removed after two and four days of storage with the result that there was a recovery in the vitamin C content of the fruit as it ripened. At the full yellow, or early eating stage, the fruit, once treated with carbon dioxide, contained as much vitamin C as fruit that had ripened in the absence of carbon dioxide.

*Garden pea.* Flats of pea seedlings grown in the greenhouse were divided into four lots in order that seedlings could be placed into treated and control tests in both light and darkness. In these tests the treated seedlings were exposed to 1 part of ethylene in 500,000 parts of air for three days while the control seedlings were held under the same conditions but without ethylene.

FIGURE 1. Color of bananas at various stages of ripening and color index numbers which correspond to those in column 2, Table III. (Plates for this figure furnished through the courtesy of United Fruit Company.)



FIGURE 1. (For description see legend on opposite page.)



Pea seedlings exposed to ethylene in the dark maintained a higher amount of ascorbic acid than untreated seedlings. The data in Table IV show this to be true also for pea cotyledons attached to the plants growing in either sunlight or darkness. If, however, the pea seedlings were grown

TABLE IV  
EFFECT OF ETHYLENE (1-500,000 PARTS OF AIR) ON THE ASCORBIC ACID CONTENT OF GARDEN PEA TREATED THREE DAYS WHILE GROWING IN LIGHT AND DARKNESS

Tissue	Light conditions	Mg. ascorbic acid per 100 g. of tissue			
		Fresh wt.		Dry wt.	
		Control	Treated	Control	Treated
Leaf and stem	Greenhouse	81	76	821	609
	Dark room	45	64	519	657
Cotyledons	Greenhouse	7	10	51	77
	Dark room	10	15	51	76

in the light during treatment with ethylene, there resulted a lower amount of vitamin C in the tissue. Since ethylene causes bending of the stem and cell enlargement with little or no further elongation of stems, one may assume that it likewise retards the loss in the dark and the accumulation in the light of vitamin C in the stems, leaves, and cotyledons. Pea seedlings growing in the dark and not exposed to ethylene lost vitamin C at a much faster rate than those seedlings retarded in growth by the ethylene. On the other hand untreated seedlings growing in sunlight accumulated vitamin C much faster than those plants exposed to ethylene. In other tests where the peas were grown entirely in the dark preceding and during treatment, a higher amount of vitamin C was found in the leaves, stems, cotyledons, and roots of the plants treated with ethylene (1-700,000 parts of air for three days) than in those held as controls. Thus one may assume that ethylene retards the fundamental processes of plant growth in the pea of which cessation of growth is the visible manifestation of the treatment.

#### DISCUSSION

Studies of the effect of various factors on the vitamin C content of asparagus shoots, pea seedlings, and banana fruit show that no general conclusion may be formed as to what will happen to the vitamin C content of a tissue under various conditions. Asparagus tissue increases in vitamin C content when the bud grows above the usual ground level, but this is not entirely an effect of light since the same result occurs when the tissue grows above ground in darkness. Storage of this tissue in 5 per cent or higher concentrations of carbon dioxide to preserve its freshness results in



the destruction of the vitamin C content and there is no recovery when the gas is removed, due, no doubt, to the fact that there is comparatively little carbohydrate reserve in the asparagus spears. Asparagus tissue has a high rate of respiration; thus one may expect large changes in the vitamin C content of the tissue, possibly in accordance with Szent-Györgyi's (12, p. 76) theory on the role of vitamin C in respiration. Bananas, on the other hand, have a comparatively lower rate of respiration and a high carbohydrate reserve; thus we might expect the changes in vitamin C content to take place at a less noticeable rate. There is a decrease in the vitamin C content with maturity and carbon dioxide can hasten this loss if the immature fruit is treated. However, unlike the asparagus tissue, the banana tissue can recover its usual vitamin C content provided the fruit has not ripened completely before the gas is removed, possibly a result of the difference in carbohydrate reserves of the two tissues. Pea seedlings respond more quickly than asparagus to light and darkness during the growing period. Also the pea seedlings show the effect of ethylene on the vitamin C content of the tissue, tending to retard both its accumulation in the light and its loss in the dark. These results suggest that possibly the vitamin C content of asparagus tissue could be preserved somewhat by treatment with ethylene. The results of the effect of ethylene treatment in maintaining a high vitamin C content of the pea cotyledons is similar to the results obtained in some cases with ripening bananas where a higher vitamin C content of the fruit was associated with previous treatment with ethylene to hasten the ripening processes. In the banana the ethylene stimulates the ripening processes and the associated carbohydrate changes have been measured; one might assume that similar changes could take place in the pea cotyledon in favor of maintaining a higher vitamin C content.

This survey indicates that the age of tissues, and the presence of ethylene and carbon dioxide affects the amount of vitamin C present. It would be well to investigate other factors or conditions of storage of plant structures in order to know what effect they may have upon vitamin C content. Hamner (4) has recently surveyed the literature on the effect of minor elements on the vitamin content of plants with a discussion of the fact that there yet remain many important problems to be solved in relation to vitamin content of plants as affected by growth factors.

#### SUMMARY

1. Asparagus buds increased in vitamin C content from 15 mg. at the dormant stage 15 cm. underground to 150 mg. per 100 g. of fresh weight when the plant was 100 cm. above ground.

2. The effect of sunlight was not entirely responsible for these results since there was a parallel increase in the bud tissue growing above the original ground level in complete darkness furnished by mounded soil.

3. Green bananas containing about 15 mg. of vitamin C per 100 g. of fresh tissue decreased to about 12 mg. when the peel was full yellow, and to 10 mg. when the peel was 50 per cent brown. The ripening colors of the bananas are shown in full color in Figure 1.

4. Carbon dioxide treatment of asparagus spears or green bananas caused a loss in the vitamin C content. Tests indicate that this loss was complete since no dehydroascorbic acid was found upon reduction with hydrogen sulphide. When the gas was removed there was no recovery of vitamin C in the asparagus, but there was complete recovery in the banana as the fruit ripened.

5. Garden peas maintained a high vitamin C content when grown in a 1-500,000 concentration of ethylene in the dark.

6. Ethylene (1-500 to 1-8000 parts of air) had no measurable effect upon the vitamin C content of the banana.

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## NON-TRANSFERENCE OF VIRUS DISEASE IN TREATMENTS OF POTATO TUBERS TO BREAK DORMANCY

F. E. DENNY

When it is intended to use the tubers from a field of potatoes as planting stock in the following year, a sample of 100 to 500 tubers is commonly taken at harvest time by reserving one tuber from each of the required number of hills properly distributed across the field. During the fall and winter months cuttings of these tubers are planted either in greenhouses, or in areas in Southern States, in order to obtain growing plants for observations as to the presence or absence of virus diseases.

These freshly-harvested tubers are in the rest period and fail to germinate when planted at once after harvest, commonly requiring a storage period of two to three months, or more, before uniform germination and thrifty growth occur. In a previous report (1) suggestions were made of methods of breaking the dormancy and of obtaining prompt growth of sprouts even within a week after harvest by the use of ethylene chlorohydrin,<sup>1</sup> ( $\text{ClCH}_2\text{CH}_2\text{OH}$ ), either alone or in combination with sodium thiocyanate ( $\text{NaSCN}$ ).

By one of these methods *intact tubers* were treated with the vapor of ethylene chlorohydrin, and it seems unlikely that under such conditions there is any possibility of a transfer of virus from one tuber to another during the process of treating. By the other method, however, *cuttings* of tubers were taken for the treatment, and since the treatment involved combining the cuttings from several tubers in one container and dipping them into a dilute solution of ethylene chlorohydrin, a virus-infected cutting could come in contact with the adjacent pieces from healthy tubers, and juice from diseased pieces, although highly diluted, would reach the surface of many others. Furthermore, in one of the methods the cuttings after having been dipped into the ethylene chlorohydrin solution were then soaked for an hour in a sodium thiocyanate solution, a process which would increase the chance for virus from an infected tuber reaching disease-free cuttings.

The object of the present experiments was to determine whether under conditions similar to those of these treatments a measurable amount of

<sup>1</sup> In the previous report (1) attention was called to the toxicity of the vapor of ethylene chlorohydrin and to precautions needed to avoid prolonged breathing of the vapor. In a recent article, Smythe and Carpenter (3) emphasize the danger of absorbing this chemical through the skin. If the chemical is accidentally spilled on the clothing, such articles should be changed at once and washed before being put on again. Rubber gloves are said not to provide adequate protection. These results were based on experiments with the 100 per cent (anhydrous) chemical.

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transfer would take place, that is, whether the count of diseased individuals in a sample would be increased by the presence of cuttings from diseased tubers. The procedure was to mix with cuttings of tubers previously shown to be healthy, cuttings of tubers previously found to be infected with virus, to carry these through the treatments, and, after obtaining well-developed plants in the greenhouse, to note whether there had been any transfer of disease to healthy plants. As controls, cuttings from each of the same tubers were planted in adjacent rows for comparison of the treated and control plants.

The first year of the test involved mosaic and leaf-roll almost exclusively, as only one spindle-tuber specimen was found in the stock of tubers available. In the second year, however, a supply of tubers each of which was known to have the spindle-tuber disease was obtained.

More than a thousand healthy cuttings were exposed to cuttings of mosaic and leaf-roll. No case of transfer of either disease to a healthy cutting was observed. The varieties Early Ohio, Bliss Triumph, Katahdin, Green Mountain, and Irish Cobbler were involved in this test. In the tests with spindle tuber only the Irish Cobbler variety was used. About 700 healthy cuttings were exposed to contact with cuttings from spindle tubers. Observations of the foliage failed to show any transfer of spindle tuber, and the plants were allowed to complete their growth in the greenhouse benches at which time the length and breadth of the tubers formed were measured. The average ratios of length to breadth were the same for the treated and the controls, and the distribution of the tubers into size groups also showed no differences.

It is believed that it is unlikely that any error in indexing for the mosaic, leaf-roll, or spindle-tuber diseases would be introduced by the use of the recommended methods of treating the dormant tubers for hastening the germination of the tubers.

#### MATERIALS AND METHODS

*Labeling of tubers.* A water-proof ink, the preparation of which was described previously (1), was used for numbering the tubers and cuttings. This allowed the sorting and identification of the cuttings after they had become mixed during the process of treating.

*Source of tubers.* The tubers (*Solanum tuberosum* L.) which were from the Institute gardens, were harvested in August of each year and were stored at 5° C. (41° F.) until needed for the treatments.

For the treatments with the crop of 1943, beginning Jan. 16, 1944, tubers were numbered individually with water-proof ink, one-quarter tuber with its number being planted in the greenhouse, and the three-quarter portion of the tuber marked with the same number being placed in a flat and held in cold storage until observations on the foliage from the planted pieces could be made. By late February the plants that developed

in the greenhouse from the planted pieces had become large enough for classification into groups, i e. either healthy, or showing the presence of mosaic, or leaf-roll, or both. Since the mosaic symptoms were distinctive and easily recognized, tubers showing both mosaic and leaf-roll were placed in the mosaic class.

For the crop of 1944, which was used for data on the spindle-tuber disease only, since no evidence of the presence of spindle tuber was found in the plants or in the tubers as shown by the relation of length to breadth, this preliminary test was not carried out and the assumption was that all tubers were healthy, at least so far as the spindle-tuber disease was concerned. Tubers known to be infected with spindle tuber were kindly contributed by Dr. E. S. Schultz, Division of Fruit and Vegetable Crops and Diseases, Bureau of Plant Industry, United States Department of Agriculture.

*Disinfection of knives.* In the experiments of 1944 with the 1943 crop the knives were placed in boiling water for one-half minute and cooled in tap water, while in the experiments of 1945 with the 1944 crop, the knives were dipped into 95 per cent alcohol, were touched with a flame, and held until the alcohol burned off. Several knives were used in rotation which permitted the knives to cool before being used again.

*Treatments.* Two methods of treatment of cut tubers were recommended in the previous report (1): (A) The "dip method," in which cuttings are placed in a container that can be closed (glass fruit jars with wide opening being the type used in these tests); covered with a dilute solution of ethylene chlorohydrin, which is then poured off at once; the dipped pieces being allowed to remain in the closed container for 16 hours at room temperature and then being planted. (B) The "dip+soak" method, in which, after the dip treatment just described has been completed, the jar is then filled with a solution of sodium thiocyanate made by dissolving 10 g. of NaSCN in a liter of water, and the treated pieces allowed to soak for one hour, after which they are rinsed thoroughly, and then planted.

Both methods were used in these tests, with the following modifications. Since the tubers were completely out of the rest period, and since small sprouts had developed by the time treatments were to begin, the dipping solution was made half-strength (15 cc. per l. instead of 30 cc.), and the dipped pieces were soaked in water instead of in one per cent NaSCN. This was done to avoid any possibility of loss of plants by injury due to the treatment.

The procedure for exposing healthy to diseased cuttings was as follows. For a comparison of the two treatment methods with a corresponding control, 25 healthy tubers (each with its original number written on it) of the proper size to furnish three cuttings were selected from the healthy stock, and two more of the identifying numbers were added, each cutting then having the same number. Three sterile wide-mouthed fruit jars were then

set on the bench and labeled to correspond to the three groups: "dip," "dip+soak," and "control." With a sterile knife three tuber cuttings were prepared, the suberized layer from the previous cut having been removed to furnish freshly cut surfaces. One cutting was placed in each jar, and this process repeated until the jars were each about one-third full. Then a diseased tuber of a size to furnish four cuttings was removed from the diseased stock, and after the suberized surfaces were removed, and the tuber was cut into four cuttings, one cutting was placed in each of the jars labeled "dip" and "dip+soak." Healthy cuttings were then added until the jars were about two-thirds full, at which time another diseased cutting was added to each of the two jars. The rest of the 25 healthy cuttings were then added to the jar. The jars marked "dip" and "dip+soak" therefore contained 25 healthy cuttings and two diseased cuttings while the "control" jar contained 25 cuttings from the same healthy tubers but without any admixture of diseased cuttings. The two jars containing two diseased cuttings each were filled with a solution made by adding 15 cc. of 40 per cent ethylene chlorohydrin to 985 cc. of water; this solution was decanted and discarded; the excess liquid was removed by inverting the jar and shaking it; a paper towel was stuffed into the neck of the jar, which was then inverted (to provide for drainage) and allowed to stand at room temperature, but not over 24° C. (75.2° F.), for about 16 hours. The control or check lot was handled in the same way except that no liquid, not even water, was added to the jar. At the end of about 16 hours the jars were opened and into the one marked "dip+soak" water was added to cover the cuttings, and the water was decanted at the end of a one-hour period of soaking.

*Planting.* All three lots were then ready for planting. The cuttings of each jar were poured on a piece of paper and placed in the order of the numbers on each cutting in rows in a flat with paper-covered bottom. A strip of a paper towel was used in picking up each cutting, and a new strip was used for each cutting. This gave three flats of cuttings, one each for the two treatments and the control, with the cutting numbers in the same order. Since the ink used in numbering the tubers was water-proof, no blurring of numbers occurred when the cuttings were planted in the greenhouse bench 6 inches apart in rows 6 inches apart. Strips of a paper towel were used in planting the cuttings, one strip for each cutting. Since the cuttings of each lot were planted in the order of the numbers on the cuttings it was easy to identify the plant from each treated cutting with the control cutting which came from the same mother tuber.

Some of the healthy tubers were of a size which permitted only two cuttings, and in such cases comparisons were made only between the "dip" method and the control, or between the "dip+soak" method and the control. But in other respects the procedure was the same as that just described.

In each experiment the diseased cuttings that were added to the jars were recovered at the end of the treatment, and these also were planted in the greenhouse in order to verify that the cutting used was in fact diseased.

Examination of the plants for symptoms of disease was delayed until full growth had been attained, approximately four to five weeks after planting.

## RESULTS

### EXPERIMENTS WITH MOSAIC AND LEAF-ROLL

These were the experiments with the crop of 1943. A total of 2125 cuttings were used of which 1275 were healthy cuttings exposed to diseased cuttings, and 850 were the corresponding controls. Irish Cobbler, Katahdin, and Early Ohio, disease-free, were tested against both mosaic and leaf-roll, while Bliss Triumph and Green Mountain were tested against mosaic only. In no case was there any transfer of disease from a diseased cutting to a healthy cutting during the process of treating.

In addition to these tests a number of experiments were carried out in which the cut surfaces of healthy and diseased cuttings were placed together and held in this position with a rubber band. The contact was maintained for 16 hours at the end of which time the two cuttings were planted at once in the greenhouse, or were placed in a beaker of water and soaked for one hour before being planted. The object of these tests was to make the contact between cut surfaces more certain than occurred in the regular treatments in which the cuttings were merely tossed into fruit jars, and in the case of the soak treatments to obtain a greater concentration of any liquid coming from the cut surface of the diseased cutting. These methods were used with 146 pairs of cuttings involving all five varieties of potatoes and the two diseases, mosaic and leaf-roll. In no case was any transmission from diseased to healthy cuttings observed.

### EXPERIMENTS WITH SPINDLE TUBER

The tubers available for tests of transmissibility of spindle-tuber disease were of the variety Irish Cobbler, crop of 1944, grown in the Institute gardens. The diseased tubers used as contaminants were of the Irish Cobbler variety, and the plants grown from cuttings of these showed that they were infected with the spindle-tuber disease by their erect foliage, acute-angled petioles, side-bend of tip leaflet, and the formation of tubers with high length-to-breadth ratio.

Fourteen lots of cuttings were employed in this test, each in groups of three, i.e., one jar of 24 cuttings treated by the "dip" method, one jar by the "dip+soak" method, both of these with exposure to cuttings with the spindle-tuber disease, and the other jar with healthy cuttings to which no diseased cuttings were added. For Lot Nos. 1 to 9 inclusive the procedure was the same as that outlined in previous paragraphs, but for Lots 10 to



14 inclusive the dipping solution was not added at once after making the cuttings; instead the cuttings were put in beakers covered with paper and allowed to stand at room temperature for four hours before the cuttings were placed in the fruit jars and dipped. Thereafter, the procedure was the same. The object of this variation was to allow the cut surfaces to dry somewhat before the liquid was added. No differences were observed be-

TABLE I

LENGTH-TO-BREADTH RATIO OF TUBERS PRODUCED BY PLANTS FROM CUTTINGS EXPOSED TO THE SPINDLE-TUBER DISEASE UNDER CONDITIONS SIMILAR TO THOSE USED IN TREATING DORMANT POTATOES TO INDUCE GERMINATION

Lot No.	Tubers from cuttings treated by				Tubers from control cuttings		Tubers from cuttings of tubers infected with spindle tuber used as contaminants	
	Dip method		Dip+soak method					
	No. tubers	Av. ratio	No. tubers	Av. ratio	No. tubers	Av. ratio	No. tubers	Av. ratio
1	36	1.0178	37	1.0530	32	1.0156	13	1.2738
2	38	1.0345	35	0.9974	43	1.0372	13	1.2285
3	40	0.9832	46	1.0035	43	1.0542	6	1.1367
4	44	1.0170	44	1.0059	37	1.0411	13	1.3115
5	45	1.0224	51	1.0328	46	0.9901	10	1.2410
6	33	1.0403	34	1.0421	19	1.0279	13	1.2315
7	22	1.0323	25	1.0412	15	0.9840	7	1.2400
8	34	0.9721	33	1.0121	26	1.0412	15	1.2407
9	25	1.0192	29	1.0300	26	1.0304	11	1.2318
10	26	0.9888	29	1.0190	46	1.0065	9	1.1544
11	46	0.9985	71	0.9935	40	1.0178	6	1.2083
12	44	1.0168	41	1.0317	43	0.9974	9	1.2233
13	53	1.0253	46	0.9922	35	1.0166	10	1.3001
14	50	1.0050	38	1.0553	45	0.9818	17	1.2088
Total Average	536 —	— 1.0123	559 —	— 1.0158	496 —	— 1.0168	152 —	— 1.2345

tween Lots 1 to 9 and Lots 10 to 14, and hereafter, in this report, no distinction is made between the two series.

The plants were examined from time to time after sprouting commenced, and comparisons were made with plants from the diseased tubers which were recovered from the treatments and planted in the same greenhouses. In no case could the presence of spindle tuber be definitely identified by examination of the foliage of plants from the lots exposed to the spindle-tuber disease by either method of treatment.

All plants were allowed to continue growth in the greenhouse benches until the tops began to die, 12 to 13 weeks after planting. Then all lots were harvested separately and measurements were made of the length and breadth of all tubers that were at least 25 mm. long. The object of these measurements was to note whether contamination with spindle-tuber cut-

tings had increased the ratio of length to breadth of tubers, which possibly could be taken as evidence of transmission of spindle tuber, even though the foliage symptoms failed to show it.

The result is shown in Table I. The lot numbers indicate the treatments that were made on the same day with exposure to the spindle-tuber cuttings, the data of which are shown in the right hand column of the same line. The bottom line shows the weighted averages of the length-to-breadth ratios. The standard deviation of the ratio among individual

TABLE II  
FREQUENCY DISTRIBUTION OF RATIOS OF LENGTH-TO-BREADTH OF TUBERS OF CROP

Range of length-to-breadth ratios	Frequencies shown by		
	Dip method	Dip+soak method	Control
1.64-1.60		1	
1.60-1.56		0	
1.56-1.52		0	
1.52-1.48		1.5	0.5
1.48-1.44		0.5	0.5
1.44-1.40		1	0
1.40-1.36		1	4
1.36-1.32	5	2	1.5
1.32-1.28	5	3	4
1.28-1.24	4	6	4
1.24-1.20	15.5	11	13.5
1.20-1.16	24.5	26.5	27
1.16-1.12	22	38.5	33
1.12-1.08	56.5	55.5	43
1.08-1.04	66.5	62.5	48.5
1.04-1.00	89	92.5	80
1.00-0.96	80.5	90	83
0.96-0.92	57	69.5	65.5
0.92-0.88	49.5	44.5	43
0.88-0.84	25.5	27	20.5
0.84-0.80	23	16	13.5
0.80-0.76	7	7	5
0.76-0.72	3.5	1.5	5.5
0.72-0.68	1	1	0.5
0.68-0.64	0		
0.64-0.60	1		
Total	536	559	496

tubers in the 42 samples represented by the treated and control lots (1591 tubers, 1549 degrees of freedom) was determined and was found to be 0.1126. There were no differences in the length-to-breadth ratios between the controls and either of the treated lots.

In order to determine whether the treatments had caused the formation of an undue proportion of tubers with high length-to-breadth ratios, frequency distributions of the ratios in the treated and control lots were made. The results are shown in Table II. In making the tests for differences

between the distributions of the control and treated lots the procedure described by Fisher (2, sect. 21) was used. Cells with small frequencies at each end of the distributions were combined, giving 13 pairs of frequencies and 12 degrees of freedom for each comparison. For the control vs. the dip method, Table II, columns 2 and 4,  $\chi^2$  was 13.45, corresponding to a probability of approximately 0.34, and for the control vs. the "dip +soak" method  $\chi^2$  was 3.02, probability approximately 0.99. Thus, there was found no indication that the treatments had increased the length-to-breadth ratios of the tubers.

As a further test of the possibility of a transfer of spindle tuber, a new crop was grown from the tubers that had been found to have high length-to-breadth ratios, in order to note whether in the next generation this characteristic would become intensified. Tubers representing each of the three groups in the upper part of Table II, after having been stored until the rest period had been completed, were planted in the greenhouse, and after about three months the length-to-breadth ratios of the tubers of this new crop were determined. The numbers of new tubers available for measurement were: 163 representing the "dip" method, 227 the "dip+soak" method, and 176 the controls. The mean length-to-breadth ratios of tubers of these three groups were 1.098, 1.111, and 1.104 in the order given, and the corresponding values for the standard deviations were 0.1239, 0.1337, and 0.1232. The new crop of tubers from plantings made of spindle-tuber diseased tubers, showed an average length-to-breadth ratio of 1.304 for 66 tubers, and a standard deviation of 0.2296.

There was, thus, no evidence of a greater length-to-breadth ratio in either of the treated lots as compared with the control. The one tuber with unusually high ratio in line 1, Table II, was cut into pieces and planted separately to see whether this high ratio would be perpetuated in the progeny. The length-to-breadth ratios of the new tubers produced from this tuber were: 0.81, 1.25, 1.09, 1.04, 1.06, indicating that the high ratio of the mother tuber was not due to the presence of spindle-tuber disease.

#### SUMMARY

In two of the methods previously suggested for treating dormant potato tubers in order to hasten germination for early observation of sprouts in the detection of virus disease, tuber cuttings from one tuber come in contact with cuttings of other tubers, with the consequent possibility that virus might be transferred from diseased to healthy cuttings in the process of treating to induce sprouting.

In order to test whether any transfer of disease occurred as a result of the treatments, cuttings from tubers known to be infected with virus disease were mixed with cuttings from disease-free tubers and these lots were then carried through the usual procedure. Observations of the plants grown

from these cuttings were then made to note whether any spread of disease had occurred.

The experiments involved exposure of more than a thousand cuttings to contamination with mosaic and leaf-roll, and about 700 cuttings to the spindle-tuber disease. The procedure was such that each plant in the treated lot could be identified and compared directly with the corresponding control plant derived from the same original tuber. In no case could it be established that there had been any transfer of virus from diseased to healthy cuttings during the process of treating.

In the case of the spindle-tuber tests, plants were allowed to continue growth until a new crop of tubers was formed, and the length-to-breadth ratios of such tubers were measured. The treated lots did not show any increase in this ratio. Samples of tubers of both treated and control lots were stored until the rest period was completed and again planted. The length-to-breadth ratios of this second crop were measured, but no differences in this ratio between the treated and check lots were found.

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# ACCUMULATION OF CARBON DIOXIDE IN POTATO TUBER TISSUE UNDER CONDITIONS FOR THE CONTINUOUS REMOVAL OF THE EXHALED GAS

F. E. DENNY

A previous paper (1) dealt with the accumulation of  $\text{CO}_2$  in tissue during an interval in which a measurement of the rate of respiration was being made, and with procedures for determining the gas content of the tissue, so that a valid estimate of the rate of respiration could be obtained in spite of the non-exhalation of part of the  $\text{CO}_2$  that was produced.

In a respiration measurement if there has been an accumulation of  $\text{CO}_2$  in the tissue the extent of this increase in internal  $\text{CO}_2$  must be taken into account, if correct values are to be found. The question then arises whether in respiration measurements as ordinarily carried out an accumulation of  $\text{CO}_2$  within the tissue is likely to occur.

When the tissue is placed in a closed container and the gaseous products allowed to accumulate in the air surrounding the tissue, conditions are favorable for an increase in internal  $\text{CO}_2$ , but in most experiments provision is made to remove the  $\text{CO}_2$  as fast as it is exhaled, either by a continuous current of air, or by the use of a layer of alkaline solution for absorbing  $\text{CO}_2$ . Does an accumulation of  $\text{CO}_2$  in the tissue take place under these conditions?

In the experiments of Whiteman and Schomer (5) a case is reported in which such an accumulation took place. When they wounded sweet potato roots, exposing an area of cut tissue and then covered over the cut surface by means of a sealing compound, there was an accumulation of  $\text{CO}_2$  within the tissues, even though there was present in the container an alkaline solution for removing the  $\text{CO}_2$  from the air surrounding the tissue.

In the present experiments even this wounding action was avoided, and only intact and uninjured tissue was used. Potato tubers which had been in storage at  $5^\circ \text{C}$ . were placed in containers and transferred to higher temperatures, and, although provision was made to remove the exhaled  $\text{CO}_2$  either in a current of air, or in addition to this, by a layer of absorbing alkali, there was an accumulation of  $\text{CO}_2$  within the tissue. At the higher temperatures ( $30^\circ \text{C}$ .,  $20^\circ \text{C}$ .) and shorter periods (up to 5.5 hrs.) more of the  $\text{CO}_2$  that was produced remained within the tissue than was exhaled from it. At the lower temperatures ( $10^\circ \text{C}$ .,  $7.5^\circ \text{C}$ .) a longer time for an accumulation of  $\text{CO}_2$  to become manifest was required (24 to 49 hrs.), but a definite increase in  $\text{CO}_2$  was obtained even when the change in temperature was only from  $5^\circ \text{C}$ . to  $7.5^\circ \text{C}$ . The error involved in neglecting to take into account the internal  $\text{CO}_2$  in estimating respiration rate under the condi-

tions of these tests varied from about 75 per cent when the change was from 5° C. to 30° C. to about 15 per cent when it was from 5° C. to 7.5° C.

### METHODS

*Tubers.* The potato (*Solanum tuberosum* L.) tubers were of the Irish Cobbler variety of the 1945 crop grown in the Institute gardens, and previous to these tests had been stored for many weeks at a constant temperature of 5° C. Small tubers were chosen in order to increase the number of tubers that could be included in a sample. The size of the sample varied from 750 to 1500 g. and the number of tubers from about 25 to 50. Duplicate samples of tubers were used for each experimental interval at each temperature.

*Apparatus.* Glass desiccators of either 2 l. or 6 l. capacity were used as containers. Removal of CO<sub>2</sub> from the desiccators as fast as formed was accomplished in the experiments at 30° C. by a current of air drawn through the desiccators by means of inlet and outlet tubes. The air current rate was approximately 450 cc. per minute and the CO<sub>2</sub> in the air stream was absorbed in Ba(OH)<sub>2</sub> solution in Van Slyke-Cullen tubes in the manner described in previous publications (1, 2, 3). Back-titration of the Ba(OH)<sub>2</sub> solution showed the amount of CO<sub>2</sub> exhaled by the sample. In the experiments at 20°, 10°, and 7.5° C., in addition to an air current of approximately 250 cc. per minute passing through the desiccator and through Van Slyke-Cullen tubes containing NaOH solution, absorption of CO<sub>2</sub> was further provided for by a solution of NaOH placed in a 15 cm. evaporating dish in the bottom of each desiccator. This solution contained 50 g. of NaOH per liter for the tests at 20° C., and 25 g. per liter for those of 10° and 7.5°. The amount of NaOH solution added to each dish was 25 to 100 cc. depending upon the duration of the experiment and the temperature. The amount of NaOH solution furnished an absorbing area of 50 to 95 sq. cm. There always remained a large excess of NaOH solution in the evaporating dish at the end of each test.

When an experiment was continued for more than 5.5 hours, the air current was stopped, and the desiccator was connected to an oxygen supply tube and this to a constant water level, so that as fast as O<sub>2</sub> was used up in the desiccator, the reduction in pressure started the water siphon and an equal amount of O<sub>2</sub> was pushed over into the desiccator. Change in temperature was not a factor since the apparatus was in a constant temperature room. In this way the normal air content of O<sub>2</sub> was maintained within the desiccator. The oxygen-supply apparatus used was a modification of that described by Whiteman and Schomer (5, p. 172). Samples of air removed from the desiccators (including those at 30° C.) at the end of the tests and analyzed with an Orsat apparatus showed that no CO<sub>2</sub> was present, and that the CO<sub>2</sub> had been removed from the air in the desiccators as fast as it was exhaled from the tubers.

The NaOH solutions in the evaporating dish in the bottom of the desiccator and in the Van Slyke-Cullen tubes were rinsed into a volumetric flask and a saturated solution of barium chloride added until no further precipitate was obtained, and, after being made up to the mark, mixed, and allowed to settle until partially clear, was titrated. The blank NaOH solution for comparison was prepared by pipetting the same amounts of NaOH solution into Van Slyke-Cullen tubes and an evaporating dish, rinsing these into a volumetric flask and adding  $\text{BaCl}_2$ . The difference between the blank and the treatment showed the amounts of  $\text{CO}_2$  exhaled from the tissue.

The  $\text{CO}_2$  content of the tissue was determined from a sample of tubers taken at the start of the experiment, at once after removal from the storage room at  $5^\circ \text{C}$ . At the end of an experimental period the tubers were removed from the desiccator and the  $\text{CO}_2$  content was again determined. The difference between these two values showed the change in the amount of  $\text{CO}_2$  in the tissue during the interval.

The apparatus used in estimating the  $\text{CO}_2$  content of the tissue was described in detail in a previous paper (1, p. 261). Briefly, the procedure was to disintegrate a weighed amount of the tissue in an alkali solution in a Waring blender, transfer to a tube fitted with inlet and outlet tubes for maintaining an air current, acidify,<sup>1</sup> and aerate out the  $\text{CO}_2$  with a current of air into  $\text{Ba}(\text{OH})_2$  solution in a series of Van Slyke-Cullen tubes. A back-titration in comparison with a blank showed the amount of  $\text{CO}_2$  in the tissue.

## RESULTS

### CHANGES IN $\text{CO}_2$ CONTENT OF TISSUE

The results are shown in Table I. The paired entries in column 4 are for the duplicate samples of tissue taken from each tuber sample, and the letters in column 3 indicate the paired tuber samples for each experimental interval. Thus, duplicate tuber samples were available for each interval at each temperature (8 pairs of duplicates in all), and these together with the five replicate starting-samples opposite zero hours in column 2 (one sample each for  $30^\circ$ ,  $20^\circ$ , and  $10^\circ$ , and two for  $7.5^\circ$ ) were combined to furnish an error term (with 12 degrees of freedom) to be used in testing for the significance of differences. The variance was found to be 7.284, which gave a standard deviation of 2.699, and a coefficient of variation of 8.6 per cent. To obtain a value to represent the  $\text{CO}_2$  contents of the tissue at the start, the values in column 4 opposite zero in column 2 were combined, giving an average starting value of 23.3. The values at each interval at each tem-

<sup>1</sup> Note error in the previous paper in the directions for the preparation of the sulphuric acid solution to be used for acidification. In Contrib. Boyce Thompson Inst. Vol. 14, No. 4, p. 262, line 32 should read 33 cc. instead of 333 cc.



perature, i.e. the values in column 5, were tested for significance by the "t" test as described by Tippett (4, sect. 5.3). In this case,  $N_1=2$  and  $N_2=5$ , giving 5 degrees of freedom as the proper line for entering the "t" table. The starred entries and the footnotes in Table I indicate the significance found.

TABLE I  
CO<sub>2</sub> CONTENT OF POTATO TUBER TISSUE AT INTERVALS AFTER TRANSFER OF  
TUBERS FROM 5° C. TO HIGHER TEMPERATURES

Temp. to which transfer from 5° was made	Hours after start	Tuber sample No.	Cc. of CO <sub>2</sub> at 0° and 760 mm. in 250 g. of tissue	Av.
30° C.	0	A	19.5 20.0	19.8
	2.5	B <sub>1</sub>	27.9 28.3	29.5*
		B <sub>2</sub>	30.2 31.5	
	5.5	C <sub>1</sub>	39.9 41.4	41.3**
		C <sub>2</sub>	42.0 41.9	
20° C.	0	A	25.8 27.0	26.4
	5.5	B <sub>1</sub>	37.8 41.6	37.9**
		B <sub>2</sub>	35.3 36.8	
	24.0	C <sub>1</sub>	50.8 50.2	49.9**
		C <sub>2</sub>	49.8 48.7	
10° C.	0	A	21.0 24.7	22.9
	5.5	B <sub>1</sub>	25.0 25.5	24.7
		B <sub>2</sub>	23.2 25.1	
	24.0	C <sub>1</sub>	29.9 27.4	30.3*
		C <sub>2</sub>	31.0 32.8	

TABLE I (Continued)

Temp. to which transfer from 5° was made	Hours after start	Tuber sample No.	Cc. of CO <sub>2</sub> at 0° and 760 mm. in 250 g. of tissue	Av.
7.5° C.	0	A <sub>1</sub>	22.8 22.9	23.8
		A <sub>2</sub>	25.1 24.2	
	25.0	B <sub>1</sub>	27.0 27.0	26.9
		B <sub>2</sub>	26.5 27.0	
	49.0	C <sub>1</sub>	30.2 29.6	30.7*
		C <sub>2</sub>	31.2 31.8	

\* "t" value greater than requirement for 0.05 prob.

\*\* "t" value greater than requirement for 0.01 prob.

At each temperature there was a gradual increase in tissue-CO<sub>2</sub> during the intervals tested. The increase was definite as early as the 2.5-hour interval when the transfer was made from 5° C. to 30° C., and also at the 5.5-hour interval for the lot at 20° C., but when the shift was from 5° to 10° a period of 24 hours and in the 7.5° series one of 49 hours were needed for the differences to become significant at the 0.05 level.

It is believed that the data in Table I show that there occurred an accumulation of CO<sub>2</sub> in the tissue of the tubers, even though the conditions were such that the exhaled CO<sub>2</sub> was removed as fast as released from the tissue.

#### RELATIVE AMOUNTS OF CO<sub>2</sub> EXHALED AND RETAINED IN TISSUE

The amounts of CO<sub>2</sub> which were exhaled and the amounts which were retained within the tissues at each temperature and each duration interval are shown in Table II. At 30°, and during the shorter interval at 20°, more of the CO<sub>2</sub> produced was retained in the tissue than was exhaled. At the longer interval at 20° and at all intervals at 10° and 7.5° more was exhaled than retained, but at the longer intervals, even at these two low temperatures, a considerable proportion of the CO<sub>2</sub> formed was retained by the tissue.

The situation in respect to the relative amounts of CO<sub>2</sub> exhaled or retained is shown in column 5. The per cent of the total amount of CO<sub>2</sub> produced which was retained by the tissue and not exhaled varied with the temperature and time. It amounted to about 75 per cent in the case of the

transfer to 30° C., 30 to 60 per cent at 20°, about 25 per cent at 10°, and about 15 per cent at 7.5°.

Columns 2, 3, and 4, Table II, also indicate the error that would be made by neglecting to take into account the CO<sub>2</sub> within the tissue in making a measurement of respiration on the basis of the CO<sub>2</sub> produced. For example, at 20°, and for the 5.5-hour interval, the respiration rate

TABLE II  
AMOUNTS OF CO<sub>2</sub> EXHALED AND RETAINED IN TISSUE ON TRANSFERENCE OF  
TUBERS FROM 5° C. TO HIGHER TEMPERATURES

Temp. to which transfer from 5° was made	Duration of test, hours	Cc. per kg. of tissue at 0° and 760 mm.		Gain in tissue CO <sub>2</sub> as % of total CO <sub>2</sub> produced
		CO <sub>2</sub> exhaled	CO <sub>2</sub> gain in tissue	
30° C.	2.5	10	44	81
	5.5	42	97	70
20° C.	5.5	31	51	62
	24.0	226	105	32
10° C.	5.5	22	8	27
	24.0	98	33	25
7.5° C.	25.0	80	13	14
	49.0	178	31	15

based on the exhaled gas alone would have been  $31 \div 5.5 = 5.64$  cc. CO<sub>2</sub>/kg./hr., whereas the rate actually occurring was  $(31 + 51) \div 5.5 = 14.90$  cc./kg./hr.

## DISCUSSION

It appears that in transferring the tubers from 5° C. to higher temperatures the rate of production of CO<sub>2</sub> within the tissue becomes greater than the rate at which it can escape into the surrounding air, even when the latter is kept CO<sub>2</sub>-free by the aerating and absorbing systems.

This difference in temperatures of transfer need not be large in order that this accumulation of CO<sub>2</sub> in the tissue will take place. This is shown by the experiment on the transfer from 5° C. to 7.5° C.

The principal cause of variation in the measurements of CO<sub>2</sub> in the tissue was due to differences between tuber samples and not to errors in the method of determining CO<sub>2</sub> in tissue samples. There were 21 pairs of duplicates of tissue samples, as shown in Table I, column 4, and the variance was found to be 1.32, giving a standard deviation of 1.15, which gives a coefficient of variation of 3.7 per cent; but even then the variation may not be entirely free of an influence due to possible inequalities in the tissue distribution to the paired lots.

The rate of respiration in the different tests can be computed by adding the  $\text{CO}_2$  values in columns 3 and 4 in Table II and dividing by the number of hours shown in column 2. The weighted average of the rates when the tubers were changed from  $5^\circ$  to  $30^\circ$ ,  $20^\circ$ ,  $10^\circ$ , and  $7.5^\circ$  were respectively 23.9, 14.0, 5.5, 4.1 cc. of  $\text{CO}_2$  (at  $0^\circ$  and 760 mm.) per kg. per hour.

### SUMMARY

Potato tubers which had been stored previously for many weeks at  $5^\circ$  C. were transferred to desiccators and placed at temperatures of  $30^\circ$ ,  $20^\circ$ ,  $10^\circ$  and  $7.5^\circ$  C. The air surrounding the tubers was kept free of  $\text{CO}_2$  by drawing a current of air through the desiccator, or, in addition to this, by absorbing the  $\text{CO}_2$  in a shallow layer of sodium hydroxide solution placed in the bottom of the desiccator. Samples of gas removed from the container at the end showed that the air surrounding the tubers was free of  $\text{CO}_2$ .

After two different duration periods at each temperature, the tubers were removed, and a determination was made of the quantity of  $\text{CO}_2$  in the tissue. By comparing these values with those for the  $\text{CO}_2$  content of the tissue at the start of the experiment, it was found that there had occurred an increase in the  $\text{CO}_2$  in the tissue. This increase became definite within 2.5 hours when the transfer was from  $5^\circ$  to  $30^\circ$ , within 5.5 hours when the temperature was  $20^\circ$ ; at  $10^\circ$ , 24 hours were required, and at  $7.5^\circ$ , 49 hours.

Analyses of the solutions of alkali used to remove the  $\text{CO}_2$  from the air current and of those in the bottoms of the desiccators showed the amount of  $\text{CO}_2$  exhaled, while the values for the tissue- $\text{CO}_2$  showed the amount of  $\text{CO}_2$  formed in the tissue but not escaping from it. When the transfer was from  $5^\circ$  to  $30^\circ$ , about 75 per cent of the  $\text{CO}_2$  produced remained in the tissue up to a duration period of 5.5 hours; when it was from  $5^\circ$  to  $20^\circ$  the tissue- $\text{CO}_2$  was about 60 per cent of the total formed up to a duration of 5.5 hours, and about 30 per cent for a period of 24 hours. For the change  $5^\circ$  to  $10^\circ$ , tissue- $\text{CO}_2$  was about 25 per cent of the total produced in a 24-hour period, and the value for the ~~5 $^\circ$  to 7.5 $^\circ$  shift was about 1.5 per cent~~ over a 49-hour period.

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# THE IDENTITY OF ALTERNARIA AND MACROSPORIUM CULTURES USED FOR SLIDE-GERMINATION TESTS OF FUNGICIDES

S. E. A. McCALLAN

A culture of *Alternaria* said to be *A. solani* (Ell. & Mart.) Jones & Grout, has been widely used in this and other laboratories for studies of fungicides by the slide-germination method (1). This culture was obtained from Dr. R. O. Magie (8) and was isolated by Dr. J. F. Adams at the University of Delaware, probably in 1935, from "collar rot" lesions on young tomato transplants from Georgia (7, footnote 5). During the intervening years the culture has become rather widely distributed under the above name. In 1941 its species identity was questioned by us because of the small size of the spores and their abundant production in culture (7, footnote 5). Various attempts to demonstrate its pathogenicity toward tomatoes in the greenhouse also have been unsuccessful. The recent studies by Groves and Skolko (2, 3) and Neergaard (10) on *Alternaria* and related genera have afforded an opportunity to scrutinize this isolate. We are indebted to Mr. J. A. Stevenson, Division of Mycology and Disease Survey, U. S. Dept. of Agriculture, Beltsville, Md. and to Mr. J. W. Groves, Division of Botany and Plant Pathology, Dept. of Agriculture, Ottawa, Canada, who have both kindly examined this culture and are agreed that it is *Alternaria oleracea* Milbraith (9), by which name it should be identified in the future.

In various papers from this laboratory beginning in 1936 (8) the organism *Alternaria oleracea* has been used under the incorrect species name of *solani*. However, it is to be noted that the true *A. solani* (Ell. & Mart.) Jones & Grout has been commonly employed by us in greenhouse studies with early blight of tomato (6), in a sporulation study (5), and in one instance in a slide-germination test (6, p. 127).

The organism known as *Macrosporium sarcinaeforme* (Cav.) is also commonly used in slide-germination tests of fungicides (1). It is probable that most of these isolates were obtained originally from Dr. J. G. Horsfall who isolated the organism from red clover (*Trifolium pratense* L.) (4). In view of the independent conclusions of Groves and Skolko (2) and of Neergaard (10) which confirm the earlier work of Wiltshire (11, 12) that the genus *Macrosporium* should be relegated to the *nomina ambigua* (the species going either to *Alternaria* or *Stemphylium*), this organism should be identified as *Stemphylium sarcinaeforme* (Cav.) Wilts. (12).

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## PARTICLE SIZE DISTRIBUTION IN COCOA POWDERS<sup>1</sup>

LAWRENCE P. MILLER

Considerable of the processing in a chocolate and cocoa plant is concerned with operations intended to reduce particle size. The importance of a velvety texture and smoothness in chocolate products has long been recognized (9, p. 96). With tissues such as cacao nib, coarseness to the palate is probably significant only when particles larger than 100 microns are present (9). In modern practice, however, other considerations in addition to the effect on the palate are important, and diameters much smaller than 100 microns are dealt with. Thus when cocoa powder is to be used in the preparation of chocolate milks in which color and stability of the suspension are major factors, the effect of particle size on the palate becomes less important but the role of particle size on color impartation and its effect on the ease with which the cocoa particles are kept in suspension require serious consideration.

It would thus appear obvious that accurate methods for the determination of the particle size distribution are desirable not only for the proper control of manufacturing operations but also to facilitate studies designed to determine the optimum particle size required to bring about the effects desired in various products in which the cocoa and chocolate are to be used. Insofar as published work indicates little advantage seems to have been taken in this industry of the methods available for the determination of particle size. Specifications are usually concerned with the fraction retained on 325 or 200 mesh sieves. With many cocoas only a small portion will be retained on a 325 mesh sieve and the material is thus essentially subsieve in size. The sieving operation, therefore, gives no information on the particle size distribution of the material (which may be over 99 per cent of the sample) which passes through the sieve. It is these fine particles, smaller than 44 microns, which have the greatest significance in many pulverized materials (2, p. 409-410). Sieving of cocoa also presents some special difficulties because of the presence of the low melting cocoa butter, and unless carefully controlled, material finer than the mesh openings may be retained on the screen in considerable amount or else excessive manipulation in attempting to get proper sieving may result in reduction of particle size during the sieving operation. In order to get an accurate picture of the particle size distribution it is necessary to use a method which obviates these difficulties and gives results which permit evaluation of the whole sample. Numerous methods for the determination of particle size are de-

<sup>1</sup> The writer is indebted to Bowey's Inc., Chicago, Ill., who supported the investigation reported herein, for permission to publish these results.

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scribed in the literature (1, 3, 4). Of these, microscopic study affords the most positive method and other methods in the main depend upon the microscope for confirmation. It is the purpose of the present paper to describe a microscopic method and to report the results of studies with some commercial cocoa powders including samples of cocoas available in retail outlets and some manufactured expressly for the preparation of chocolate drinks. Preliminary studies made on the particle size distribution in chocolate liquor and cocoa powder produced in the same plant have shown the cocoa to be the coarser. It would thus appear that the cocoa cake disintegrator does not succeed in breaking down the cake to the degree of fineness attained in the original milling.

The microscopic method used and the calculations and interpretations of the results obtained are based on the publications of Hatch (5) and Hatch and Choate (7) with modifications introduced to permit greater accuracy in the range of the larger particles. These large particles, while relatively few in number, are very important on a weight basis since weight is a function of the cube of the diameter. Briefly stated these authors showed that if measurements of individual particles are arranged in groups of equal size intervals and the percentage frequency of each group is plotted against the mid-value of the sizes included, such a size-frequency distribution curve will follow the normal probability curve, when the logarithms of the sizes are substituted for the sizes themselves. This fact enables the data to be plotted to form a straight line when the percentage of particles below a certain size is plotted against the size using logarithmic probability paper (8). The geometric mean,  $M_g$ , can be obtained from the straight line by reading the 50 per cent point. The constant  $\sigma_g$ , called the standard deviation, which is a measure of the dispersion of the observations from the mean, can be calculated from readings taken from this line. These two statistical parameters,  $M_g$  and  $\sigma_g$ , define one and only one size frequency curve. Various average diameters which are important in evaluating the samples can be calculated from  $M_g$  and  $\sigma_g$  by using the formulae developed by Hatch (5) and Hatch and Choate (7).

#### METHOD FOR MEASURING DIAMETERS OF INDIVIDUAL PARTICLES

In samples in which the diameters cover a wide range of size, measurements of individual particles and calculation of the arithmetic mean may put undue emphasis on the smaller particles which are the least significant on a weight basis since weight is proportional to the cube of the diameter. Thus, if one had a sample containing 99 per cent of particles 10 microns in diameter and 1 per cent of particles 50 microns in diameter, measurement of several hundred particles for the construction of a line representing the powder as a whole would not properly evaluate the 1 per cent by number comprising over 50 per cent by weight. In such cases it is usually

suggested that there be some prior separation by sieves and determination of the distribution in the several fractions. However, as pointed out above, separation of cocoa powder by sieves is not very satisfactory. In Figure 1 is shown a surface photograph, magnification  $\times 135$ , of powder remaining on a 325 mesh screen after careful sieving at  $-5^{\circ}\text{C}$ . It can readily be seen that small particles in large numbers cling to the particles larger than the 44 micron screen openings. Therefore a procedure was developed, described below, in which the larger particles were measured in much greater number than indicated by their frequency. Greater precision for the points at the upper end of the curve, where most of the weight lies, was thus attained.

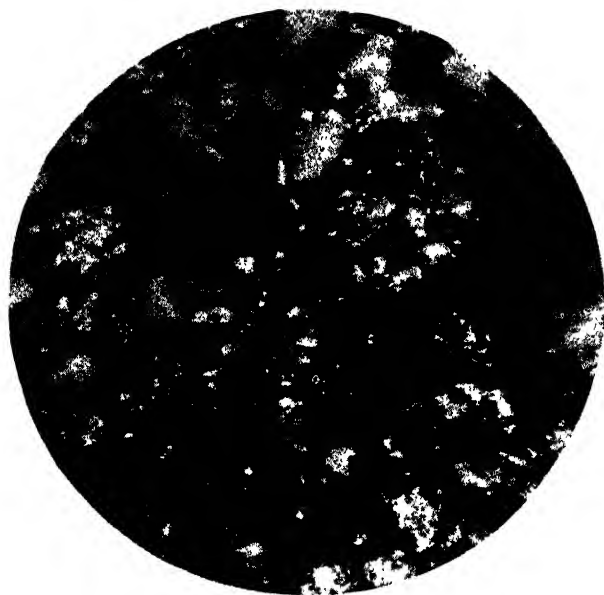


FIGURE 1. Surface photograph of cocoa powder retained on a 325 mesh screen.  $\times 135$ . Note the large number of smaller particles clinging to the particles larger than 44 microns.

For the measurements the cocoa powder was mounted on microscope slides in oil of cloves. The oil of cloves and powder were carefully mixed to avoid, as much as possible, any artificial agglomeration of particles. The slide was placed in a microscope fitted to project the image on a ground glass screen. The magnification of the particles as seen on the screen was determined with a stage micrometer and in the experiments reported here was 910 diameters. A portion of the area of the screen was marked out and the largest horizontal diameter of every particle in the area for a number of fields was measured with a rule graduated in millimeters. Usually 250 particles were measured. The values were then converted to microns and divided into groups differing from each other by the  $\sqrt{2}$ . The data were

plotted (open circles) on logarithmic probability paper as shown in the upper curve in Figure 2.

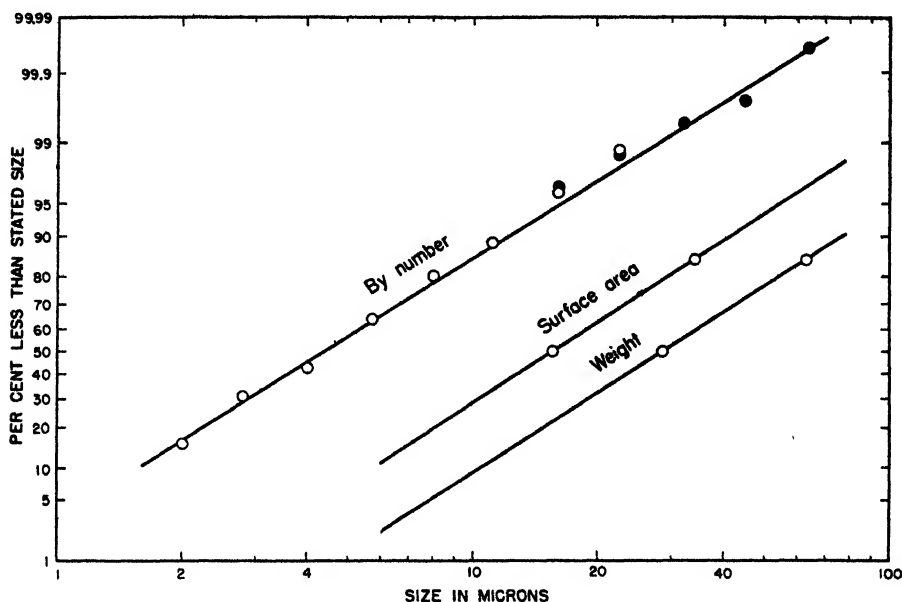


FIGURE 2. Particle size distribution by number, by surface area, and by weight plotted on logarithmic probability paper. Brand A of Table I.

To obtain better values for the upper part of the curve, additional measurements were made of the size distribution of particles 16 microns in diameter and larger. The per cent by number of such particles was determined with greater accuracy by counting 1500 particles on the screen. With the aid of a microscope equipped with an ocular micrometer 100 particles, 16 microns and larger, were measured, the data divided into groups differing by  $\sqrt{2}$  and based on the percentage of these particles in the sample additional points (represented by closed circles) plotted. The effect of this procedure was the same as if it had been possible to separate out from the powder particles 16 microns and larger and to measure 100 of such separated particles. If a given powder contained 1 per cent of such particles and a procedure of measuring every particle was followed it would have been necessary to measure 10,000 particles before as many as 100 would have been covered in this range. In drawing the best straight line, by eye, representing the points plotted an attempt was made to allow for the increased accuracy of the points in the upper range, also the comparatively good values around the 50 per cent point and the lesser precision of the points on the lower part of the line.

## CALCULATION OF VARIOUS AVERAGE DIAMETERS

Having obtained a straight line representing the particle size distribution, the geometric mean,  $M_g$ , can be obtained directly by reading the 50 per cent point. The standard deviation,  $\sigma_g$ , is given by

$$\frac{84.13\% \text{ size}}{50\% \text{ size}}.$$

From the statistical parameters,  $M_g$  and  $\sigma_g$ , the following average diameters can be calculated using the equations developed by Hatch (5).

Average diameter	Symbol	Mathematical definition, antilog	Equation
Arithmetic mean	$\delta$	$\frac{\sum nd}{\sum n}$	$\log M_g + 1.1513 \log^2 \sigma_g$
Specific surface	$d_s$	$\left(\frac{\sum nd^{-1}}{\sum n}\right)^{-1}$	$\log M_g - 1.1513 \log^2 \sigma_g$
Surface area	$\Delta$	$\left(\frac{\sum nd^2}{\sum n}\right)^{1/2}$	$\log M_g + 2.3026 \log^2 \sigma_g$
Volume	$D$	$\left(\frac{\sum nd^3}{\sum n}\right)^{1/3}$	$\log M_g + 3.4539 \log^2 \sigma_g$
Surface area per unit volume	$\frac{D^3}{\Delta^2}$	$\left(\frac{\sum nd^3}{\sum nd^2}\right)$	$\log M_g + 5.7565 \log^2 \sigma_g$

It is immediately apparent that the use of the equations greatly simplifies the calculations that would otherwise have to be made to solve for the expressions given in column 3. It is essential that at least some of these average diameters be considered in evaluating a powder, such as cocoa powder, with a wide range in particle size since in such cases, the most obvious average diameter, that is, the arithmetic mean, has little physical meaning.

It is possible also, from  $M_g$  and  $\sigma_g$  to determine the geometric mean by surface area  $M_g^A$  (6), and the geometric mean by weight,  $M_g'$ , and to construct lines showing the distribution on a surface area and on a weight basis. It is then possible to read off diameters above or below which certain percentages of weight or surface area lie. In considering such data it should be kept in mind that in changing from count diameters to weight diameters the data are cubed and any errors are therefore greatly magnified. Nevertheless this procedure seems useful in giving physical meaning to the data

and in emphasizing the rather considerable differences obtained with various samples of cocoa powders.  $M_g'$  may be obtained from the expression  $\log M_g' = \log M_g + 6.9078 \log^2 \sigma_g$  and  $M_g^\Delta$  from  $\log M_g^\Delta = \log M_g + 4.6054 \log^2 \sigma_g$  (6). By solving the equation

$$\sigma_g = \frac{84.13\% \text{ size}}{50\% \text{ size}}$$

for the 84.13 per cent size using the values  $M_g'$  and  $M_g^\Delta$ , respectively for the 50 per cent size and the  $\sigma_g$  by count, the second point for each line is obtained and the two lines, representing distributions by surface area and by weight can be drawn. Such lines are shown in Figure 2 for the cocoa powder concerned with therein.

### RESULTS

*With cocoa powders.* In Table I are shown the results obtained with a number of cocoa powders purchased at retail stores. Values for  $M_g$  and  $\sigma_g$  as obtained from the plots on logarithmic probability paper are given together with the values for a number of other average diameters calculated

TABLE I  
PARTICLE SIZE DISTRIBUTION IN SOME COCOA POWDERS AVAILABLE AT RETAIL

Brand	Geo-metric mean, $M_g$	Stand-ard deviation, $\sigma_g$	Arith-metic mean, $\delta$	Spe-cific sur-face, $d_s$	Sur-face area, $\Delta$	Vol-ume, $D$	Sur-face area per unit vol., $\frac{D^3}{\Delta^2}$	Sur-face area mean, $M_g^\Delta$	Weight mean, $M_g'$	Uni-formity coefficient*
A	4.49	2.19	6.11	3.30	8.30	11.3	20.9	15.4	28.4	3.30
B	3.83	2.43	5.68	2.69	8.43	12.5	27.5	18.5	40.8	3.86
C	4.29	2.54	6.62	2.78	10.2	15.8	37.7	24.4	42.8	4.11
D	3.48	2.53	5.35	2.26	8.24	12.7	30.0	19.5	46.2	4.17
E	3.28	2.59	5.16	2.09	8.11	12.8	31.6	20.1	49.6	4.21

\*  $\frac{60\% \text{ size}}{10\% \text{ size}}$  on weight basis.

as outlined above. In addition the uniformity coefficient, obtained by dividing the 60 per cent size by the 10 per cent size as determined from the weight curves, is shown in the last column. Similar data for cocoas manufactured for use in chocolate drinks are given in Table II. It is readily seen that the particle size distribution varies widely with different brands and with different samples of the same brand. In general the cocoas prepared for use in chocolate drinks were finer than the powders on sale at

TABLE II  
PARTICLE SIZE DISTRIBUTION IN COCOA POWDERS MANUFACTURED  
FOR USE IN CHOCOLATE DRINKS

Brand and sample number	Geometric mean, $M_g$	Standard deviation, $\sigma_g$	Arithmetic mean, $\delta$	Specific surface, $d_s$	Surface area, $\Delta$	Volume, $D$	Surface area per unit vol., $\frac{D_s}{\Delta^2}$	Surface area mean, $M_g \Delta$	Weight mean, $M_g'$	Uniformity coefficient*
F	3.00	2.08	3.92	2.30	5.13	6.71	11.5	8.77	15.0	2.91
G <sub>1</sub>	3.07	2.34	4.41	2.14	6.33	9.08	18.7	13.0	26.9	3.64
G <sub>2</sub>	3.55	2.14	4.74	2.66	6.33	8.46	15.1	11.3	20.2	3.21
G <sub>3</sub>	3.38	2.23	4.66	2.45	6.43	8.87	16.9	12.2	23.3	3.39
G <sub>4</sub>	3.75	2.25	5.21	2.70	7.24	10.1	19.4	14.0	27.0	3.46
G <sub>5</sub>	4.99	2.14	6.67	3.73	8.90	11.9	21.2	15.9	28.3	3.14
G <sub>6</sub>	3.77	2.29	5.31	2.68	7.49	10.6	21.0	14.9	20.6	3.48
H	2.33	2.52	3.57	1.52	5.48	8.39	19.7	12.9	30.2	4.13
I	3.63	2.40	5.33	2.47	7.81	11.5	24.7	16.8	36.2	3.81

\*  $\frac{60\% \text{ size}}{10\% \text{ size}}$  on weight basis.

retail stores. Values obtained for the diameter above which 50 per cent of the weight lies in the cocoas of Tables I and II varied from 15 microns to 49.6 microns. It is thus apparent that in operations where particle size is important some method of control is essential for efficient operation.

*With chocolate liquors.* Since two grinding operations (grinding of the nib to chocolate liquor prior to extraction of some of the fat and pulverization of the resultant cocoa cake) are involved in the manufacture of cocoa powder it is important to know something about the relative contribution of the two processes to the fineness of the powder produced. Preliminary data only have been obtained on this point. An examination of eight chocolate liquors gave an average of 2.46 for  $M_g$  and 2.31 for  $\sigma_g$  while six cocoa powders made in the same plant from similar but not the identical liquors had an average  $M_g$  of 3.75 and an average  $\sigma_g$  of 2.24. The corresponding geometric means on a weight basis correspond to 20.2 and 26.4 microns. These results suggest that the cocoa pulverizer did not succeed in breaking up the cake to the fineness attained in the milling of the liquor and that to produce finer cocoa in the case of the equipment used, emphasis should be placed on increased efficiency of the cocoa pulverizer unit.

*Photomicrographs of cocoa powders.* Frequently in cases in which a complete study of the particle-size distribution does not seem to be warranted, much information can be gained by examination of the powder under a microscope. When the difference between two samples of cocoa is considerable this is readily apparent upon such examination. For some purposes

this may be sufficient. For a permanent record photomicrographs can be taken. Care must be taken that the particular field chosen is representative of the powder. If a sufficient number of fields are photographed and the powder is mounted properly, measurements of the size of the individual particles can be made from the photomicrographs.



FIGURE 3. Photomicrograph of a coarse cocoa powder mounted in oil of cloves.  $\times 500$ . Size of squares is equivalent to the openings of a 325 mesh screen magnified 500 times.

When the photographs are desired for a matter of record only, a magnification of 500 diameters is useful. It is also helpful to superimpose on the photograph of the particles lines drawn in such a way that the openings between them are squares of the same size as the openings of a 325 mesh screen magnified 500 times. The presence of particles larger than these openings can then be readily detected. Two such photomicrographs are shown in Figures 3 and 4.

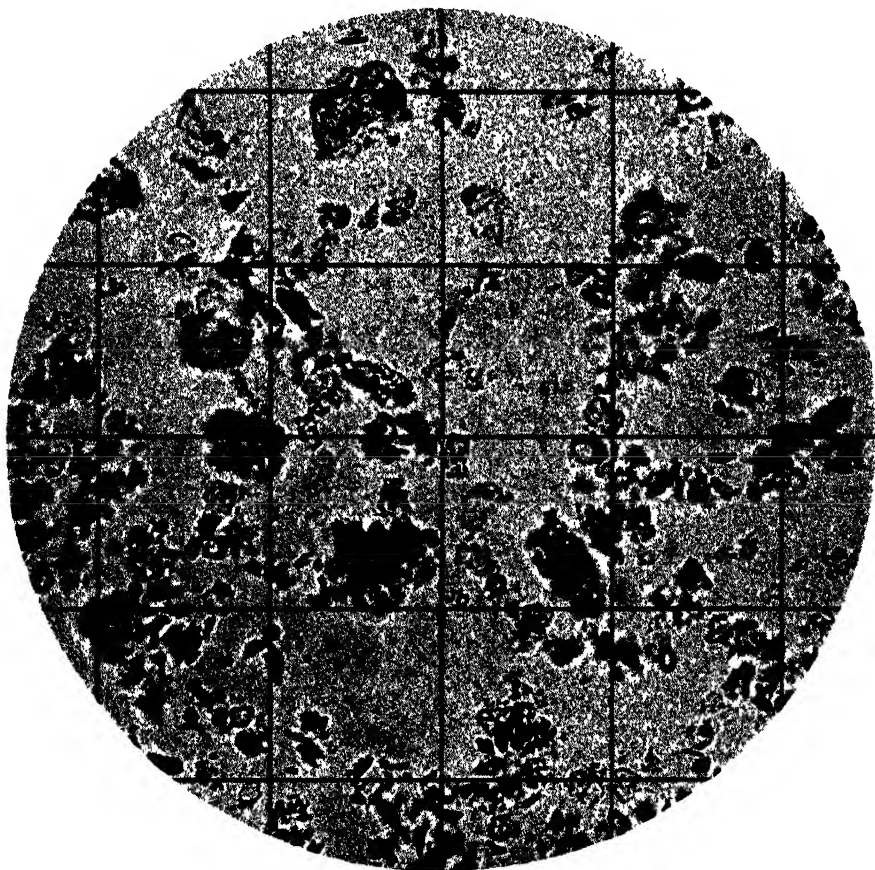


FIGURE 4. Finely ground cocoa in oil of cloves.  $\times 500$ .

#### SUMMARY

A method is described for the measurement of the size distribution and calculation of various average diameters for cocoa powders. This method is based on the work of Hatch and Hatch and Choate whereby the plotting of the data on logarithmic probability paper results in a straight line from which the geometric mean,  $M_g$ , and the standard deviation,  $\sigma_g$ , can be determined and other average diameters calculated from these values. A modification giving greater accuracy in the range of the larger particles without physical separation of these particles, which is difficult in the case of cocoa powder, has been introduced.

The particle size distribution was determined in a number of cocoa powders sold in retail stores and in some cocoas manufactured expressly for use in chocolate drinks. The results show that the commercially availa-



ble powders differ markedly in particle size and therefore emphasize the need for control of particle size in cocoas that are to be used in products the quality of which is affected by particle size.

Preliminary results indicate that in ordinary commercial practice the cocoa pulverizer does not succeed in breaking down the cocoa cake to as fine a state of subdivision as has been attained in the chocolate liquor prior to the pressing operation.

Particle size evaluation by ordinary screening is inadequate since modern fine cocoas are largely subsieve in size and furthermore sieving is difficult to carry out properly because of the presence of the low-melting cocoa butter.

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# A RAPID METHOD FOR THE EVALUATION OF THE LARGER PARTICLES PRESENT IN COCOA POWDERS<sup>1</sup>

LAWRENCE P. MILLER

A method for the determination of the particle size distribution of cocoa powders involving actual measurements of the diameters of individual particles, plotting of the data on logarithmic probability paper, and the calculation of various average diameters is described in a previous paper (1). In connection with the manufacture and use of cocoa powder it is desirable to have available a method somewhat simpler than the above and yet of sufficient dependability to be of use in the commercial evaluation of the quality of the powders concerned. In commercial practice emphasis is usually placed on the fractions larger than 325 or 200 mesh. As pointed out in the preceding paper sieving of cocoa powder is rather unsatisfactory and it is desirable to be able to detect and evaluate large particles by a method more reproducible than sieving. In certain processes the user of cocoa powder does not seem to be particularly concerned with the particle size distribution of the powder smaller than 200 mesh, but is much interested in the presence of particles larger than 200 mesh (74 microns) since such particles may cause trouble in the finished product. By observing the settling rate of the largest particles (which, of course, settle most rapidly) in a long glass tube in a suitable medium, it has been possible to detect the presence of unusually large particles in a given sample of cocoa powder and measurements of the rate of fall of such particles give an evaluation of the size of the particles concerned. Much experience with this type of test has shown that the results are easily reproducible and that differences between cocoa powders can readily be measured. It thus serves as a rapid quantitative method for the comparative evaluation of cocoa powders with respect to the largest particles in the powders.

It is the purpose of this paper to present the details of the apparatus and procedure in the hope that others interested in particle size in cocoa powders, or other powdered materials, may use the method or some modification of it. Examples of the results obtained are also given.

## THE SETTLING TUBE

The apparatus consists of a long glass tube with a 1 cc. pipette graduated in hundredths sealed to the lower end. The tubing has an outside diameter of 7 mm. and an inner diameter of about 5.5 mm. The total length, and therefore the height of the column of liquid traversed by the particles, is 144 cm. Of this the lower 32 cm. consists of the graduated

<sup>1</sup> The writer is indebted to Bowey's Inc., Chicago, Ill., who supported the investigation reported herein, for permission to publish these results.

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pipette. An adapter is attached by means of a small piece of rubber tubing to the upper end of the tube and a pinch clamp is supplied which on opening permits the fall of the particles, previously added to the adapter above the clamp, to begin. The lower end of the tube is closed off with a tightly fitting rubber policeman.

#### METHOD OF MAKING A DETERMINATION

The tube is first filled with the settling medium which was usually 95 per cent ethyl alcohol or Solox (a commercially available type of denatured alcohol) to the level of the pinch clamp. The sample is prepared by weighing out 300 mg. and dispersing the cocoa by shaking for about one minute in a mixture of 5 cc. of petroleum ether and 4 cc. of 95 per cent alcohol. This is then added to the adapter above the pinch clamp, the clamp opened, and the time measured by means of a stop watch for the first ten particles to fall to the bottom of the tube. With some fine cocoas it is not possible to count ten particles before the arrival of a cloudy mass at the bottom of the tube. In these cases the time required for the settling of the mass of particles is taken. With the aid of the graduated pipette it is also possible in some cases to measure the time required for various fractions of the powder to settle and thus construct a sedimentation curve for the whole powder. With some samples, for reasons not entirely clear at this time, a definite line of demarkation between the settled powder and the still falling particles was not evident. In those cases a curve for the whole powder could not be obtained.

#### RESULTS

The average time required for the first ten particles to settle in the samples of cocoa examined during the course of these tests varied from 1.33 for the coarsest cocoa to 13.45 minutes for a cocoa especially finely ground. In Table I are listed some determinations in quadruplicate on a number of samples. The purpose in giving this table is to illustrate the type of results obtained. For these determinations the 300 mg. samples were dispersed in 9 cc. of alcohol instead of the mixture of 5 cc. of petroleum ether and 4 cc. of alcohol recommended above. The use of alcohol alone is quite satisfactory in most cases but with unusually finely ground cocoas better dispersion is obtained with the petroleum ether alcohol mixture and it is therefore recommended for routine use.

An analysis of variance of the data given in Table I, excluding the results with brand K, which is obviously coarser than any of the other samples, shows that a difference between totals of 2.04 is required for a significance of 20:1 and 2.75 for 100:1 significance.

In Table II are shown the results obtained with known mixtures of a coarse (settling time, 3.35 minutes) and a finely ground cocoa (settling time, 10.13 minutes). The finely ground sample had been passed through

TABLE I

TIME OF SETTLING IN MINUTES OF FIRST 10 PARTICLES IN 300 MG. SAMPLE  
OF VARIOUS COCOA POWDERS IN 144 CM. TUBE IN  
95 PER CENT ETHYL ALCOHOL

Brand	Settling time, minutes	Total for four detns.
K	1.08, 1.46, 1.36, 1.42	5.32
L, as received	4.73, 4.57, 4.70, 4.35	18.35
L, fraction > 200 mesh	3.69, 3.70, 3.73, 3.76	14.88
L, fraction < 200 mesh	7.73, 7.97, 7.53, 7.72	30.95
M	6.77, 6.83, 6.84, 7.30	27.74
M <sub>1</sub>	6.62, 6.33, 6.38, 6.59	25.92
M <sub>2</sub>	7.36, 7.16, 8.37, 7.86	30.75
M <sub>3</sub>	7.35, 8.18, 8.42, 8.37	32.32
M <sub>4</sub>	9.80, 9.12, 9.49, 9.22	37.63
M <sub>5</sub>	9.99, 9.13, 9.65, 9.39	38.16

a 325 mesh screen and the coarse cocoa was material retained on a 200 mesh screen. The data indicate that about 0.15 per cent of the coarse fraction has to be present before a significantly faster settling rate of the first ten particles becomes evident. The data when plotted as shown in Figure 1 give a fairly smooth curve which might serve as a calibration curve in tests with unknown mixtures. Such a curve, however, will probably apply only to the two cocoas in question and could not be used as a general calibration curve.

TABLE II

SETTLING TIME IN MINUTES OF FIRST TEN PARTICLES IN A 300 MG. SAMPLE  
OF KNOWN MIXTURES OF FINE AND COARSE COCOAS

Coarse fraction, %	Settling time, minutes	Total for four detns.
100	3.35	—
10	4.83, 4.61, 5.16, 4.66	19.26
5	5.59, 5.38, 5.42, 5.72	22.11
2.5	6.37, 5.85, 5.89, 6.26	24.37
1.25	7.58, 7.40, 7.26, 6.77	29.01
0.65	7.75, 7.59, 6.71, 8.46	30.51
0.325	8.54, 8.83, 8.48, 9.27	35.12
0.163	8.38, 8.33, 8.82, 9.47	35.00
0.08	11.18, 10.11, 9.97, 10.19	41.45
0.04	10.26, 10.73, 11.06, 10.18	42.23
0.00	9.44, 10.54, 9.94, 10.61	40.53

### INTERPRETATION OF RESULTS

In considering the data obtained in measuring the time for the first ten particles to settle, it is of course necessary to keep in mind that only a

small fraction of the powder is involved and the method gives no information as to the size distribution in the rest of the sample. Thus it is possible for a powder to give a fast settling rate for the first ten particles and yet,

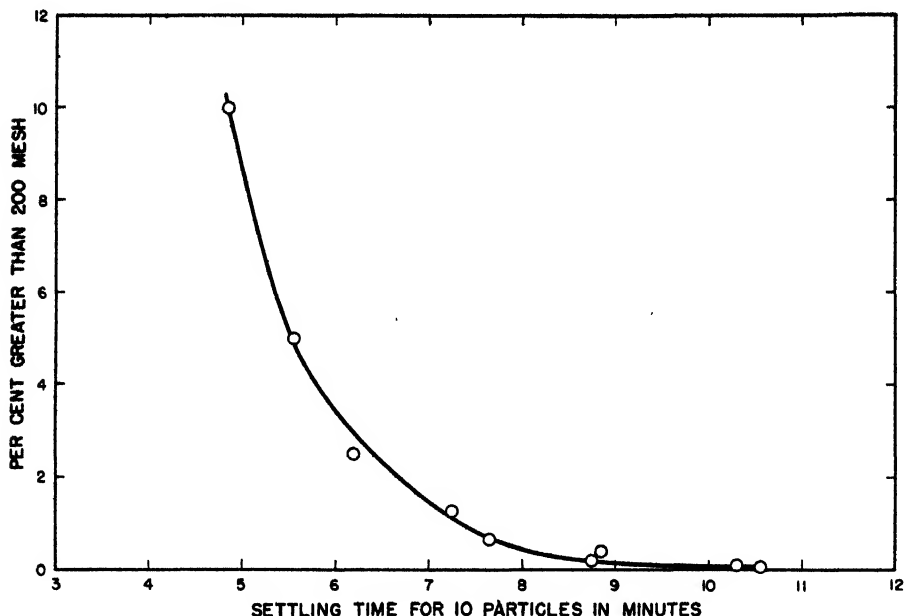


FIGURE 1. Relation between per cent of  $>200$  mesh cocoa in a powder and settling time for the first ten particles.

taking the powder as a whole, be more finely ground than another sample having a slower rate for the first ten particles. What is perhaps an extreme case is illustrated by two of the samples included in the data of Table I. The sedimentation curves for brand K, time for the first ten particles, 1.33 minutes, and for brand L, 4.59 minutes, are shown in Figure 2. It is clear that brand K, aside from containing a small fraction of very coarse material is really a finely ground cocoa, while brand L does not contain particles as large as the largest of brand K, but nevertheless is a coarse cocoa taking the sample as a whole. Differences in the character of the samples as a whole were readily confirmed by examination under the microscope. These results with brand K and L serve to emphasize that the settling rate of the first ten particles is a measure only of the coarsest portion of the sample in question.

#### DETERMINATION OF PARTICLE SIZE BY THE APPLICATION OF STOKES' LAW

It can be seen from the curves of Figure 2 that in cases where the rate

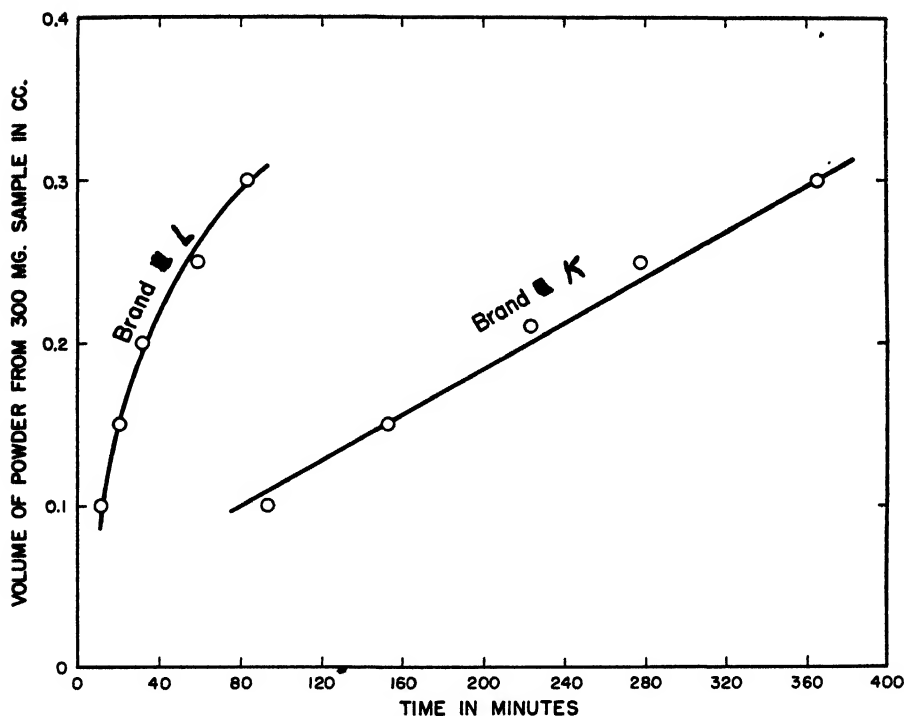


FIGURE 2. Curves showing the settling rates of two cocoa powders.

of settling of the whole sample is measured it is possible to read off from the curve the time required for one-half of the sample by volume to settle. One can calculate the diameter of the particles at this midpoint with the aid of Stokes' Law which may be formulated as follows:

$$d^2 = \frac{18\eta h \times 10^8}{(D_s - D_1)gt}$$

where

$\eta$  = viscosity of medium in poises, 0.012

$h$  = height of tube in cm., 144 minus height of settled powder at the midpoint

$D_s$  = density of particles, 1.40

$D_1$  = density of medium, 0.80

$g$  = gravitational constant in cm. per second per second, 980

$t$  = time of fall in seconds

$d$  = diameter in microns

In a number of cases the geometric mean diameter by weight,  $M_g'$  (1), had been determined on powders also evaluated by the settling method. The mean diameters at the 50 per cent point by volume as determined by

the application of Stokes' Law to the settling data show fair agreement with the values for  $M_g'$ . The values for the two constants would not be expected to be identical since the finer powder no doubt packs more closely than the coarser so that one would expect the value for  $M_g'$  to be the higher of the two. This was the case in four out of the five instances where both constants were determined. Values for five cocoas as obtained from Stokes' Law were 42, 37, 57, 32, and 27 while the corresponding figures for  $M_g'$  were 50, 43, 46, 41, and 28 respectively.

#### SUMMARY

A rapid method for evaluating the coarser particles present in cocoa powder is described. This involves the measurement of the rate of settling of the first ten particles in a 144 cm. tube filled with 95 per cent alcohol. Data are given showing the reproducibility of the method and illustrating the effect of the presence of various percentages of a coarse fraction in a finely ground cocoa.

It is also shown that by measuring the time required for various fractions of the powder to settle (with aid of a graduated pipette forming the lower part of the settling tube) the particle diameter corresponding to the 50 per cent point by volume can be determined with the aid of Stokes' Law.

It is suggested that evaluation of a cocoa powder, or other powders, by measuring the time required for the first ten particles to settle, should be useful in cases in which primary interest is concerned with the larger particles present and in which more involved studies of particle size distribution are not feasible.

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# SOME COMPOUNDS RELATED TO THE INSECTICIDE "DDT" AND THEIR EFFECTIVENESS AGAINST MOSQUITO LARVAE AND HOUSEFLIES

EDWARD A. PRILL, MARTIN E. SYNERHOLM, AND  
ALBERT HARTZELL

Since the outstanding insecticidal activity of 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane ("DDT") has become generally known, the synthesis and testing of related compounds have been actively pursued in many laboratories with the object of finding insecticidal compounds possibly better than "DDT" itself, particularly compounds with faster knockdown ability, less toxicity toward warm-blooded animals, less phytotoxicity, greater specificity of action against harmful rather than beneficial insects, and with more nearly ideal physical properties; as well as the object of finding correlations between chemical structure and insecticidal activity.

At Boyce Thompson Institute for Plant Research, during the past three years, compounds related to "DDT" have been synthesized and tested as insecticides against mosquito larvae and houseflies. The compounds and their data are given in Table I; and additional test data on those compounds which were found effective against houseflies are given in Table II. From these laboratories data on "DDT" and four of its alkoxy analogs have been published (34) and a repetition of some of these data (indicated in the tables) is included here to facilitate comparison with closely related compounds.

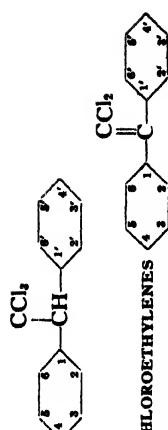
## PREPARATION OF COMPOUNDS

Each symmetrical analog of "DDT" was made by the Baeyer reaction (1) of chloral hydrate with an aromatic compound. (The formula of the aromatic compound used in each case is readily made apparent by replacing the trichloroethylidene group by hydrogen atoms in the formula of the analog.) In the monosubstituted benzenes used, the substituent was an *ortho-para* orienting group such as a halogen, a thiocyno group, a methyl group, a hydroxyl group, an alkoxyl group, or a trichlorovinyl group, and by analogy to many reported similar reactions it may be assumed that the linkage established between the substituted phenyl radical and the trichloroethylidene radical was predominantly *para* to the substituent, and recrystallization of the product obtained should yield the pure *p,p'* (4,4') analog. In most of the polysubstituted benzenes used as intermediates, at least one of the substituents was an alkoxyl group; and it may be assumed, in view of the fact that an alkoxyl group has a stronger orienting effect than a halogen or a methyl group, that the linkage established was

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TABLE I  
COMPOUNDS RELATED TO "DDT"



Type	Compound	M.p. (uncorr.) ° C.	Chlorine content, %*		Results of tests		References††	
			Calcd.	Found	Mosquito larvae, rating**	Houseflies, concn. in- effective, g. per 100 ml.***	Chemical	Entomological
A	None	64			C	†	1, 16	5, 9, 24, 28, 36, 37
—HCl	None	80			D	2.0	2	25
A	4,4'-Dichloro (DDT) †	107			A	†	40	28, 29
—HCl	4,4'-Dichloro	80			B	1.0	40	5, 9, 24, 36
A	4,4'-Difluoro	139			A	†	21, 25	9, 21, 25, 37
A	4,4'-Dibromo	173			B	†	40	25, 37
A	4,4'-Diiodo	234-235	26.6	26.7	D	0.2	6	25
A	4,4'-Dithiocyano	80			A	†	11, 16	5, 9, 22, 25, 36, 37
A	4,4'-Dimethyl	74-75	42.3	41.6	B	†	6	25
A	4-Chloro	94			A	†	39	9, 22, 24, 37
A	4-Chloro-4'-methyl	206			E	1.0	12, 16	5, 9, 22, 25, 34, 36, 37
A	4,4'-Dihydroxy	89			A	†	12	25
—HCl	4,4'-Dimethoxy †	109			E	0.5	12, 16	34, 37
A	4,4'-Dimethoxy	105			A	†	34, 37	34, 37
A	4,4'-Diethoxy †	84	29.6	29.4	A	†	34, 37	34, 37
A	4-Methoxy-4'-ethoxy	62	26.5	26.0	C	†	34, 37	34, 37
A	4,4'-Di- <i>n</i> -propoxy †	50	24.8	24.6	E	0.5	34, 37	34, 37
A	4,4'-Di- <i>n</i> -butoxy †	75	24.5	24.1	D	1.0	34, 37	34, 37
A	4,4'-Bis(β-methoxyethoxy)	Oil (crude)	16.7	16.4	E	0.5	34, 37	34, 37
A	4,4'-Bis(β-ethoxyethoxy)	60	54.4	54.4	B	†	34, 37	34, 37
—HCl	4,4'-Bis(trichlorovinyl)	118	28.3	27.8	B	0.5	34, 37	34, 37
A	4,4'-Dimethoxy-3,3'-dimethyl	99	19.4	19.8	B	0.5	34, 37	34, 37
A	4,4'-Diethoxy-3,3'-dimethyl	Gum (crude)						
—HCl	4,4'-Diethoxy-3,3'-dimethyl	97						

TABLE I (Continued)

Type	Compound	Substituents	M.p. (uncorr.) ° C.	Chlorine content, %		Results of tests		References††	
				Calcd.	Found	Mosquito larvae rating**	Houseflies, concn. in- fective, g. per 100 ml.***	Chemical	Entomological
A	4,4'-Diethoxy-3,3'-dichloro		Gum (crude)	34.9	34.7	B	†		
-HCl	4,4'-Diethoxy-3,3'-dichloro		99			E			
A	4,4'-Dimethoxy-2,2'-dimethyl		Gum (crude)	21.0	22.1	E	1.0		
-HCl	4,4'-Dimethoxy-2,2'-dimethyl		104	26.3	26.5	E	0.5		
A	4,4'-Dimethoxy-2,6,2',6'-tetramethyl		133	26.2	26.1	D	1.0	37	37
A	3,4,3',4'-Tetramethoxy		120	19.2	19.8	E	0.5		
-HCl	3,4,3',4'-Tetramethoxy		87-88	29.6	29.4	A	†		
A	3,4-Methylenedioxy-4'-methoxy		153-155	46.4	45.8	E	0.5	37	37
-HCl	2,2'-(or 5,5')-Dimethyl-5,5'-(or 2,2')-dichloro††		140	40.7	39.9	E			
A	2,2'-Dimethoxy-5,5'-dichloro		146	42.6	42.1	E	0.5		
A	2,2-Dimethoxy-5,5'-dimethyl		158	28.3	27.6	E	0.5	16	
A	2,2-Diethoxy-5,5'-dimethyl		128	26.5	26.2	E	2.0	16	
-HCl	2,2-Diethoxy-5,5'-dimethyl		134	19.3	18.4	E			
A	2,5,2',5'-Tetramethoxy		115	26.2	26.6	E	0.5	37	37
A	2,5,2',5'-Tetraethoxy		125	23.1	23.1	E	0.5		
A	2,2-Bis(2-thienyl)-1,1,1-trichloroethane		74-75				1.0	33	13

\* The chlorine analyses (Parr bomb) were performed by Victor Cullmann.

\*\* The effectiveness of a compound against mosquito larvae (*Culex quinquefasciatus* Say) is arbitrarily rated as follows: A—LD<sub>50</sub> of 0.01 to 0.05 p.p.m. (parts per million); B—LD<sub>50</sub> of 0.05 to 0.2 p.p.m.; C—LD<sub>50</sub> of 0.2 to 1.0 p.p.m.; D—LD<sub>50</sub> of 1.0 to 4.0 p.p.m.; E—No or negligible kill at 4.0 p.p.m.

\*\*\* Solutions of the compounds with 0.025 g. added pyrethrins, or in some cases with 1.0 ml. of added "Lethane 384" per 100 ml. to provide a knockdown, were tested against houseflies (*Musca domestica* L.) by the Peet-Grady method. Since in many cases the compound contributed practically nothing to the knockdown or kill at the concentration tested, the concentration used is simply indicated herein. Data for the more active compounds (indicated by †) are presented more fully in Table II.

†† See Literature Cited. Only the more pertinent references are cited. When no reference number is given the compound has not been mentioned in the literature as far as the authors are aware at the time this is written.

‡ Data from these laboratories on these compounds were previously published (34) and are included here for comparison.

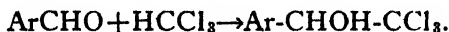
†† This substance made from *p*-chlorotoluene and chloral is probably a mixture. Stephenson and Waters (37) obtained a product melting at 142° C. from these intermediates which they considered as the (?) 2,2'-dichloro-5,5'-dimethyl analog.

*para* to the alkoxy group whenever this position was unoccupied; otherwise *ortho* to it.

The symmetrical analogs containing alkoxy substituents were made by the reaction of two moles of the appropriate aryl ether per mole of chloral hydrate in the presence of an acetic acid-sulphuric acid mixture in essentially the manner described by Fritsch and Feldmann (12) for the diethoxy analog but with the modification of cooling the reaction mixture in ice. Attempts to prepare sulphur-containing analogs similarly from phenyl alkyl thio ethers were unsuccessful.

Trichlorovinyl phenyl ether (27), and phenyl thiocyanate, as well as the benzene derivatives not containing a strongly activating hydroxyl or alkoxy substituent, were each condensed with chloral hydrate by means of concentrated sulphuric acid alone.

In making the unsymmetrical analogs the intermediary carbinols were prepared through adaptations of the method studied by Howard (20), wherein an aromatic aldehyde reacts with chloroform in the presence of powdered potassium hydroxide, as shown in the following general equation:



[Such carbinols, corresponding to the intermediary product of the Baeuer reaction, are also known to be formed by using a large excess of chloral in proportion to the aromatic compound and using sulphuric acid (6) or anhydrous aluminum chloride (8) as the condensing agent.] The carbinol made from *p*-chlorobenzaldehyde (20) was condensed by means of concentrated sulphuric acid with benzene to produce the 4-chloro analog and with toluene to produce the 4-chloro-4'-methyl analog. Using a cold acetic acid-sulphuric acid mixture as a condensing agent the carbinol made from anisaldehyde was condensed with phenetole to produce the 4-methoxy-4'-ethoxy analog. Similarly, the carbinol made from piperonal was condensed with anisole to produce the 3,4-methylenedioxy-4'-methoxy analog.

Attempts to prepare the 4-chloro-4'-methoxy and the 4-chloro-4'-ethoxy analogs by reaction of the carbinol made from *p*-chlorobenzaldehyde with anisole and phenetole, respectively, in cold acetic acid-sulphuric acid mixture, yielded as the only crystallizable product in both cases, the acetate of the carbinol (20), which was identified by melting point (122° C.) and mixed melting point with an authentic sample of this derivative. Other attempts to prepare these analogs using sulphuric acid alone or aluminum chloride as condensing agent were unsuccessful.

By condensation of chloral hydrate with an equimolar mixture of phenetole and anisole in the presence of cold acetic acid-sulphuric acid mixture a crude product was obtained, which theoretically should consist of the 4-methoxy-4'-ethoxy, 4,4'-diethoxy, and 4,4'-dimethoxy analogs in approximately the ratio of 2:1:1. This product was tested (Table II only) without recrystallization.

TABLE II  
RESULTS OF THE PEET-GRADY TESTS ON HOUSEFLIES OF THE MORE  
EFFECTIVE "DDT" ANALOGS

Analog where substituents are	Concn. g. per 100 ml.	Pyreth- rins g. per 100 ml.	10-min. knock- down %	24-hr. kill %	OTI kill %
None	2.0 4.0	0.025 0.025	97 99	37 73	42 58
4,4'-Dichloro (DDT)*	0.25 0.1 0.1	0 0.025 0.05	42 85 96	38 73 86	49 59 44
4,4'-Difluoro (crude)	0.2 0.5 1.0	0.025 0.025 0	91 92 99	42 88 99	46 55 60
4,4'-Dibromo	0.1	0.025	97	83	48
4,4'-Diiodo	0.2 1.0	0.025 0.025	97 86	44 83	47 56
4,4'-Dimethyl	0.5 0.5 1.0	0 0.025 0.025	0 86 99	— 56 66	43 43 42
4-Chloro	1.0	0.025	93	48	42
4-Chloro-4'-methyl	0.25 0.5	0.025 0.025	83 85	37 78	42 56
4,4'-Dimethoxy*	0.2 0.4	0 0	99 99	32 85	34 46
4,4'-Diethoxy*	0.2 0.1	0 0.05	90 97	86 78	34 44
4-Methoxy-4'-ethoxy	0.3 0.3 0.15	0 0 0.025	95 96 93	85 85 69	41 53 53
Mixture of 4,4'-diethoxy and 4,4'-dimethoxy	0.15 + 0.15 0.15 + 0.15 0.075 + 0.075	0 0 0.025	92 95 90	88 91 71	41 53 53
Crude product from phenetole-ani- sole mixture and chloral	0.3	0	96	93	41
4,4'-Di-n-propoxy*	0.5	0.025	95	48	54
4,4'-Dimethoxy-3,3'-dimethyl	0.5 0.5 2.0	0 0.025 0.025	0 94 86	— 63 83	43 52 56
4,4'-Diethoxy-3,3'-dichloro (crude)	0.2 1.0	(1.0 ml. "Lethane 384") 0.025	96 89	29 89	44 48
3,4-Methylenedioxy-4'-methoxy	0.125 0.125 0.25 0.25	0 0.025 0 0.05	93 97 96 99	76 74 86 87	47 47 46 47
Pyrethrins alone (typical results)		0.025 0.05	84 93	21 32	50 50

\* Previously reported (34).

Dehydrochlorination products of some of the analogs were prepared by refluxing with alcoholic KOH for several hours. In several cases where the parent analog was non-crystalline, the isolation and characterization of a pure crystalline dehydrochlorination product served to establish the identity of the major component of the parent substance.

Each crystalline compound was recrystallized at least once from dilute solution in 95 per cent ethyl alcohol. The non-crystalline products were precipitated as oils or gums from alcoholic solution on cooling or on dilution with water and cannot be considered as pure compounds, particularly in view of the corresponding case of technical "DDT," which is a mixture of isomers often containing as little as 70 per cent of the *p,p'*-"DDT" (14).

#### TEST METHODS

*Mosquito larvae.* Solutions of the compound were tested against 5- or 6-day-old culicine mosquito larvae (*Culex quinquefasciatus* Say)<sup>1</sup> by the method previously described by Hartzell and Wilcoxon (18). In testing a compound, a known amount dissolved in acetone was added to Yonkers tap water and progressive dilution by a factor of two made with water. The concentration of acetone in the strongest solution was not greater than 1.5 ml. per liter. As a rule, the compounds were not tested at concentrations greater than 4 parts per million (p.p.m.) because usually this exceeded the solubility of the compound in water. Mortality counts were made after about 20 hours. The data on mosquito larvae given in Table I represent the results obtained with at least two cultures of the larvae.

From their studies of "DDT" as a larvicide, Eide, Deonier, and Burrell (10, p. 541) state "Many of the culicine species are more resistant to DDT than are the anopheline." It is apparent that the results obtained with one species of mosquito larvae are not necessarily applicable to another species.

*Houseflies (Musca domestica L.).* Solutions of the compounds were prepared in a highly refined kerosene ("Deo-base") with, when necessary, a small quantity of acetone as co-solvent. Usually a small amount of added knockdown agent such as 0.025 g. pyrethrins per 100 ml. or 1 ml. "Lethane 384"<sup>2</sup> per 100 ml., was added. The tests were made by the large group Peet-Grady method (32).

The percentage kill given by the Official Test Insecticide (OTI) (31), which contains approximately 0.2 g. pyrethrins per 100 ml., was determined for each culture of flies used; and this provides an index of the resistance of the culture toward pyrethrum. The variations in resistance of cultures toward pyrethrum and toward other insecticidal compounds are

<sup>1</sup> Mosquito eggs were obtained from Orlando, Florida, via air mail through the courtesy of Dr. C. H. Bradley.

<sup>2</sup> A commercial solution, 50 per cent by volume, of  $\beta$ -butoxy- $\beta'$ -thiocyanodiethyl ether in purified kerosene.

not parallel, and this is especially true in the case of "DDT" and its effective relatives because of the difference in the mode of action of "DDT" (22, 23) as compared with that of pyrethrins, and as indicated by the histological effects (17) produced. OTI kills that are within the allowable limits show at least that the cultures are normal.

Admittedly, Peet-Grady tests do not measure the full potentialities of "DDT" and its effective relatives, in particular with respect to residual effect. However, since space sprays are in demand, results obtained by the Peet-Grady method are of some interest as a means of comparison. Many of the published data on the effectiveness of "DDT" and related compounds against houseflies were obtained by methods other than those based on space sprays. Results obtained by allowing the flies to walk on a surface which has been treated with an insecticide and those obtained by exposing the flies to an insecticidal spray for a limited period of time, will be somewhat different.

#### DISCUSSION OF RESULTS

*Mosquito larvae.* The analog with no substituent in either ring showed considerable activity toward mosquito larvae, being about one-tenth as effective as "DDT."

The most effective analogs comprised the symmetrically 4,4'-disubstituted analogs in which both substituents were chlorine, fluorine, methyl, methoxyl, or ethoxyl; and also the following unsymmetrical analogs: the 4-chloro-4'-methyl, the 4-methoxy-4'-ethoxy, and the 3,4-methylenedioxy-4-methoxy analogs. On the basis of the tests performed it was not possible to establish the relative order of effectiveness of these most active analogs and "DDT" toward the larvae. Somewhat less active mono- and disubstituted analogs were the 4-chloro, 4,4'-dibromo (only slightly less active than "DDT"), 4,4'-diiodo, and the 4,4'-di-*n*-propoxy analogs.

The 4,4'-dihydroxy analog was relatively inactive, possibly because of its low solubility in lipids.

The other 4,4'-disubstituted analogs, such as the 4,4'-di-*n*-butoxy, 4,4'-bis( $\beta$ -methoxyethoxy), 4,4'-bis( $\beta$ -ethoxyethoxy), and the 4,4'-bis(trichlorovinyl)oxy analogs, each of which contains substituents of large size (volume), showed little or no activity.

Disubstituted analogs having methoxyl or ethoxyl substituents in the 4,4' positions were about as active as "DDT," but such analogs further substituted in the 3,3' positions by methyl groups or chlorine atoms were "grade B" in activity. Some of the tetra and more highly substituted analogs, although containing methoxyl substituents in the 4,4' positions, showed little or no activity.

The substituted analogs which contain no substituent in either the 4 or 4' position were inactive. (The examples given here were made from

*p*-disubstituted benzene derivatives and consequently no position *para* to a substituent was unoccupied and available for linkage to the trichloroethylidene radical.)

The dehydrochlorination product of "DDT" was found to be about one-tenth as active as its parent compound. This relationship also held in the case of the dehydrochlorination product of the unsubstituted analog. The dehydrochlorination product of the 4,4'-dimethoxy analog, however, showed no activity.

*Houseflies.* The analog with no substituent in either ring was found only slightly active toward houseflies when used at a concentration of 2 g. per 100 ml. in the Peet-Grady test. It is reported (22) that this simplest member was the first of the "DDT" family studied by the Swiss investigators. It could well have happened that after only a Peet-Grady test with this compound on houseflies, it might have been dismissed; and by analogy, possibly also the entire "DDT" family, as being insufficiently active.

The analogs which were found to be effective in about the same range as "DDT" in the tests on mosquito larvae were also found effective in the Peet-Grady tests on houseflies; certain differences, however, in respect to type or degree of effectiveness showed up in the latter tests.

The 4,4'-difluoro analog, which had been used by the Germans as the active ingredient of "Gix," apparently has as its chief advantage over "DDT," the property of being liquid [the highly purified compound, however, is reported to have a melting point as high as 45° C. (21, 25)] and thus more easily emulsifiable in water. In the Peet-Grady tests in "Deobase," it did not show as great a killing power as "DDT." The 4,4'-dibromo analog appeared almost as active as "DDT" but the 4,4'-diiodo analog was much less active. The 4,4'-dimethyl, the 4-chloro, and the 4-chloro-4'-methyl analogs were only moderately active.

As has been reported (34) from these laboratories, the 4,4'-dimethoxy analog has good knockdown ability—a property not possessed by "DDT" at comparable concentrations; however, its killing power is considerably less than that of "DDT." The 4,4'-diethoxy analog has a killing power about two-thirds that of "DDT" and a knockdown ability better than that of "DDT"; although, in the latter respect it is inferior to the dimethoxy compound.

The 4-methoxy-4'-ethoxy analog was practically indistinguishable in its action from a mixture of equal parts of the 4,4'-dimethoxy and 4,4'-diethoxy analogs. The same can be said of the crude product made from the phenetole-anisole mixture and chloral.

Since the 4,4'-di-*n*-propoxy and the 4,4'-di-*n*-butoxy analogs were found (34) to have a low order of activity, it was considered of interest to study alkoxy analogs, the alkoxy groups of which contain three or four carbon atoms and in addition an oxygen atom interrupting the carbon

chain. In the 4,4'-bis( $\beta$ -methoxyethoxy) and the 4,4'-bis( $\beta$ -ethoxyethoxy) analogs (Table I), the additional oxygen conferred no improvement.

The 3,4-methylenedioxy-4'-methoxy analog was found to be of considerably greater effectiveness against houseflies than "DDT" both in respect to knockdown and kill. It is well known that certain compounds containing a methylenedioxyphenyl radical in their structures (15, 38)—but not all such compounds (15, 35)—are active as insecticides, and particularly as synergists with pyrethrum. Possibly this compound owes its exceptional activity to both its relationship to "DDT" and its possession of a methylenedioxyphenyl radical in its structure. It is significant to note here that the synergism with pyrethrins so often encountered among compounds possessing the methylenedioxyphenyl group does not show up in the case of this compound. It is also significant that a somewhat closely related analog without a methylenedioxy group, namely, the 3,4,3',4'-tetramethoxy analog was inactive against houseflies as well as against mosquito larvae.

In view of the fact that certain organic thiocyno compounds are effective insecticides, it was considered of interest to investigate the 4,4'-dithiocyno analog (Table I). No activity of this compound was found in the tests on houseflies. Moreover, the extremely low solubility of this particular compound is to its disadvantage.

The 4,4'-dimethoxy-3,3'-dimethyl and the 4,4'-diethoxy-3,3'-dichloro analogs were found to be only moderately active in the Peet-Grady tests. The substituents in the 3,3' positions apparently are responsible for the diminished activity in accord with the observation noted above in the case of the mosquito larvae tests.

Undoubtedly, some of the other analogs which were moderately active against mosquito larvae would also show some activity against houseflies if tested at much higher concentrations than are reported in Table I.

The dehydrochlorination products tested against houseflies showed no activity against this insect.

#### GENERAL DISCUSSION

The alkoxy analogs are of particular interest. Of the analogs of this type dealt with in this paper, the following are of possible practical importance as insecticides: (A) The 4,4'-dimethoxy analog, (B) the 4,4'-diethoxy analog, (C) the 4-methoxy-4'-ethoxy analog; or better, a product containing this as well as some of the previous two symmetrical analogs (such a product can be easily made from an anisole-phenetole mixture and chloral, and it may have certain advantages, such as, increased solubility), and (D) the 3,4-methylenedioxy-4'-methoxy analog, especially if a more economical method for its preparation is available. Studies on the rates of dehydrochlorination by alkali in the case of the 4,4'-dimethoxy (3, 7)



and the 4,4'-diethoxy<sup>(3)</sup> analogs show that these are more stable than "DDT." Greater stability toward alkaline matter is a very desirable property in connection with many insecticidal uses. Presumably, certain of the active alkoxy analogs also would be more stable than "DDT" under the conditions of storage of aerosol bombs. The liberation of hydrogen chloride from "DDT" causes deterioration (19) of the bombs.

The following generalizations on the relationship between chemical structure and effectiveness against the insects can be drawn from the present work. Symmetrical 4,4'-disubstituted analogs in which the substituents are halogen, methoxyl, ethoxyl or methyl groups are active. The examples studied suggest that an unsymmetrical analog with substituents in the 4 or 4,4' positions has an activity intermediate between the activities of the two symmetrical compounds of which it may be considered a hybrid; however, many more unsymmetrical analogs will have to be studied, particularly those with very dissimilar substituents, before a definite statement can be made on this point. When the substituents in the 4,4' positions are larger than a certain critical size (volume rather than weight apparently is the more important), the analog has little or no activity. Analog having methoxyl or ethoxyl substituents in the 4,4' positions and also additional substituents, such as methyl, chlorine, or methoxyl in other positions are less active than the analogs having only the methoxyl or ethoxyl substituents in the 4,4' positions. Tetrasubstituted analogs with no substituent in the 4 or 4' position, such as the analogs made from chloral and *p*-alkoxymethylbenzenes, *p*-dialkoxybenzenes, or *p*-chloromethylbenzene, are inactive.

In attempts to explain the activities of "DDT" type compounds, theories based upon certain properties of the compound, such as the ability to readily lose hydrogen chloride (26), or the presence of a "lipoid soluble group" in their structures (22, 30), have been proposed. There are too many exceptional cases to allow consideration of the existence of either of the above mentioned properties as a sufficient condition for activity; the existence of both properties may be a necessary condition for activity. The compound apparently must have certain substituents in the 4,4' positions in order to have a high degree of activity. Martin and Wain (25) point out that no apparent relationship exists between the electronic effects (electromeric and inductive effects) of the substituents in the 4,4' positions and the toxicity of the compound. According to the more recent concepts (13, 23), the spatial relationship of the different parts of the molecule to each other is the most important factor.

The effect on insects is not the only subject that may be considered. Insects are perhaps most susceptible because the compounds can easily enter their nervous system and vital organs. Many of the compounds are also more or less toxic to warm-blooded animals (9, 23)—a fact which

necessitates care in their use. Possible effects on living matter in general constitute an open field for study. In this connection may be mentioned the recent report (4) that the 4,4'-diamino and the 4,4'-dinitro analogs of "DDT" show promise as possible chemotherapeutic agents.

#### SUMMARY

Thirty-two compounds of the "DDT" type are described and compared with respect to their effectiveness against mosquito larvae (*Culex quinquefasciatus* Say) and houseflies (*Musca domestica* L.). Among these are several unsymmetrical analogs.

Ten of these have been converted to their dehydrochlorination products.

Of the analogs of "DDT," only a few alkoxy analogs were found really superior to "DDT" with respect to either knockdown ability or kill when used against houseflies or in their effectiveness against mosquito larvae.

From the data at hand, it appears that a compound of the "DDT" type must have certain substituents in the 4,4' positions in order to have a high degree of activity. The presence of additional substituents in other positions appears to cause a diminution of the activity.

About half of the compounds dealt with are new and these, if crystalline, have been characterized by analyses.

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## SPECIAL STUDIES ON SEED COAT IMPERMEABILITY

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### INTRODUCTION

That the seeds of many leguminous plants possess impermeable coats is a well established fact. From the point of view of continuance of the species, this is a very desirable trait. Not only do such seeds remain viable for long periods of time, but, under natural conditions, individual seeds become permeable at different periods after harvest so that any one lot of seeds is capable of producing seedlings over a period of several years. However, this same character has made it difficult to obtain reliable tests of the germination capacity of a given lot of seeds and to secure good commercial seedling stands. In addition to the economic importance of many legumes, the scientific facts to be discovered in permeability studies have led to much investigation.

Among the known methods devised for making the hard coats permeable are mechanical scarification, concentrated sulphuric acid treatment, boiling water, and special temperature treatments. The literature on the subject has been reviewed adequately by several writers. Only the reports directly concerned with the present studies will be presented here.

The weather factors effective in opening the coats of sweet clover (*Melilotus*) have been studied by Martin (5). He found that, in natural seeding in the field or in dry storage over winter in unheated open buildings in Iowa, 80 to 100 per cent of the hard seeds softened by the middle of the following April. In spite of the fact that practically all of the opening of the seed coats to the absorption of water occurred in the interval from March 20 to April 20, a previous exposure of two months or more to fluctuations of temperature near the freezing point was required for effective softening. A constant temperature of 10° C. as well as higher fluctuating temperatures were ineffective in making the seed coats permeable.

Freezing has been found to reduce the number of impermeable alfalfa seeds (6). After the first freezing, however, subsequent freezing and thawing had little effect. Also the intensity of the freezing was without effect within the range of 0° to -20° C. Busse (1) reported that freezing air-dry impermeable seeds of sweet clover and alfalfa in liquid air (-190° C.) made them permeable. Sweet clover seeds were kept in liquid air for 175 days without injury. Cooling to -80° C. softened some of the impermeable alfalfa seed but had little effect on sweet clover seeds. Busse attributed the increased germination after freezing to formation of very tiny cracks in the impermeable membrane. The very low freezing temperature required for

rendering hard sweet clover seeds capable of moisture absorption explains the ineffectiveness of freezing and thawing in soil plantings under natural weather conditions.

In 1932, Hamly (2) described a new method for making hard seeds of sweet clover (*Melilotus alba*) permeable. This consisted in shaking the seeds for ten minutes at three oscillations per second in a 500 cc. corked Florence flask. Special mechanical impactors were also used. This treatment caused the formation of a stropholar cleft through which water could enter. Hamly stated that the permeability of naturally soft seeds also occurred through the opening of a cleft at the strophiole. Scarification and sulphuric acid treatment, on the other hand, produced unlocalized permeable areas in the hard seed coat. The permeable areas of the coats were determined by osmic acid staining. This shaking method was later applied by Hutton and Porter (3) for several different species of legumes and found effective.

Examination of the structure and nature of the seed coat following sulphuric acid treatment in five species of legumes led Rees (7) to list the impermeable portions thus: for *Indigofera arrecta*, *Cytisus albus*, and *Acacia melanoxylon*, the cuticle; for *Melilotus alba* the cuticularized ends of palisade cells; for *Albizzia lophantha* the cuticle and palisade cells. The impermeability was attributed to the presence of cutin in some part of the seed coat. Boiling in absolute alcohol made the coats of all of these seeds permeable but since water at the same temperature had the same effect it was considered a direct result of the temperature.

Verschaffelt (8) made an extensive study of the effects of some chemical treatments on hard-coated seeds. His main work was done with seeds of *Gleditsia triacanthos*. Soaking some of these seeds for 24 hours in absolute alcohol caused subsequent swelling in water. Different lots of the same species required only two or three hours' soaking in alcohol to render their coats permeable. Other species also responded to this alcohol treatment. Seeds of all members of the subfamily Caesalpinoideae and most of those of Mimosoideae which Verschaffelt studied took up water readily after immersion in alcohol. In a large majority of cases he thought these effects were not produced by the extraction of a water-proofing substance since other chemicals with similar capacities for dissolving such substances were without effect. He explained rather that the alcohol was able to enter the integument in the hilum region through interstices through which water cannot ordinarily pass. That water then followed the path of the penetrated alcohol was shown by using aqueous solutions of dyes. Hard coats of seeds of members of the subfamily Papilionoideae were not affected by alcohol treatment.

The conclusions of Verschaffelt minimize the importance of the cuticle in causing impermeability of seed coats. According to Lute (4) the cuticle

of alfalfa seeds does not prevent the absorption of water. These seeds were observed to swell only when the tips of the palisade cells were broken.

The present tests were undertaken to determine whether hard-coated seeds made permeable by shaking would also be affected by soaking in alcohol and vice versa. Also the possible importance of phylogenetic relationships in such responses was considered.

#### MATERIAL AND METHODS

Seeds were selected to represent three subfamilies of the family Leguminosae. *Acacia aneura* F. V. M., *A. constricta* Benth., *A. decurrens* Willd., *A. greggii* Gray, *A. hakeoides* A. Cunn., *A. linifolia* Willd., *A. pycnantha* Benth., *A. saligna* Wendl., *Leucaena pulverulenta* Benth., and *Prosopis velutina* Wooton, belong to the subfamily Mimosoideae. From the Papilionoideae, *Cytisus scoparius* (L.) Link., *Melilotus alba* Desr., *Robinia pseudo-acacia* L., *Cladrastis lutea* (Michx. L.) Koch., and *C. amurensis* Koch. were selected. Representative of Caesalpinioideae were *Cassia artemisoides* Gaud., *C. leptocarpa* Benth., *Cercidium floridum* Benth., *Gleditsia triacanthos* L., *G. triacanthos* L. var. *inermis* Pursh., *Gymnocladus dioica* (L.) Koch., *Parkinsonia aculeata* L., *P. microphylla* Torr., *Cercis canadensis* L., and *C. chinensis* Bunge. Most of the seeds were obtained through the courtesy of Mr. Fred Gibson of the Southwestern Arboretum, Superior, Arizona. Others were collected in the vicinity of Yonkers, N. Y.

After preliminary tests in which the seeds were soaked in absolute ethyl alcohol for periods of 2, 4, 8, 16, 24, 48, and 72 hours, the last named period was selected as being the most effective. Preliminary tests for shaking determined twenty minutes as an effective time. For shaking tests the seeds were placed in a glass bottle of approximately 240 ml. capacity fitted with a rubber stopper. The diameter of the bottle used was approximately two and one-half inches and the height three inches up to the neck of the bottle. The bottle was placed in a reciprocating oscillating machine and shaken for twenty minutes. The bottle moved in a horizontal direction at a rate of approximately 400 two-inch strokes per minute.

Most of the data which will be presented here will be the results of the alcohol and shaking tests. Other trials for making the seed coats permeable included soaking in 95 per cent and 50 per cent instead of absolute alcohol, ether, xylol, carbon tetrachloride, benzol, and acetone. None of these substances was as effective as absolute alcohol for making seed coats permeable. To test the effects of detergents, seeds were placed in Vatsol OT (1 part per 1000), Ultrol (0.5 per cent), and Tergitol Penetrant (0.4 per cent) where they were left for 2, 4, 8, 16, 24, and 48 hours, after which they were placed in water. No swelling of seeds above that of the untreated controls occurred. These latter tests were used for *Gleditsia triacanthos* and



*Melilotus alba* only. Dry seeds of *Gleditsia* and *Melilotus* were also subjected to chlorine gas at two atmospheres pressure for one and two days; at three atmospheres pressure for four days; and at five atmospheres pressure for thirty minutes. No effect of these treatments was noted for intact or filed seeds, i.e., hard coats were not made permeable and seeds with filed coats were not injured.

Samples of all types of seeds were boiled in water and in 1 per cent sodium bicarbonate [ $\text{Na}(\text{CO}_3)_2$ ] solution for two or five minutes. These methods, especially the sodium bicarbonate solution, produced swelling but in most cases the swollen seeds failed to germinate and rotted, indicating injury. However, a satisfactory boiling treatment could probably be determined for each variety.

Limited tests using liquid nitrogen were made and will be described below. Also the effects of winter and spring plantings on seed coat permeability will be presented.

Throughout the experiments, intact and filed seeds were used as controls. Seeds were filed individually by hand or by holding against sandpaper revolved at high speed by an electric motor.

#### RESULTS AND DISCUSSION

Table I contains data on effects on seeds of alcohol soaking and shaking as compared with the filed and intact seeds used as controls. The species have been arranged according to their subfamily relationships. When one compares the Papilionoideae and the Caesalpinioideae, it becomes evident immediately that the seeds reacted as a group. Seeds of the members of the Papilionoideae (*Cladrastis*, *Cytisus*, *Melilotus*, and *Robinia*) were made permeable by shaking in a glass bottle for twenty minutes. None of these seeds gave more swelling after soaking in absolute ethyl alcohol for 72 hours than the corresponding intact controls. Seeds of the Caesalpinioideae subfamily, on the other hand, were made permeable by the alcohol soaking but not by the shaking. An exception were seeds of *Parkinsonia microphylla*, 90 per cent of which became swollen after shaking for twenty minutes. By prolonging the shaking period to forty minutes a few more seeds of this group were made permeable. Doubtless more shaking would finally abrade the coats so that they would absorb water.

The differential responses of these two subfamilies to the two types of treatment indicate two types of causes of failure to absorb water. One, characteristic of the Papilionoideae, is the strophiolar cleft described by Hamly (2). Hutton and Porter (3) reported shaking effective for five species of legumes all of which belong to this same subfamily. They examined shaken seeds of *Amorpha fruticosa* L. and *Lespedeza capitata* Michx. and found fissures at the base of the hilum depression through which water entered the seed. As far as the author is aware, references in

the published literature to effective shaking methods have all been the result of tests with seeds of Papilionoideae, though this fact has not been stressed.

On the other hand, the beneficial effect of alcohol soaking on seeds of Caesalpinoideae and its failure to make seeds of Papilionoideae permeable was reported in detail by Verschaffelt (8). Whether the mode of entry of water into alcohol treated seeds is through interstices in the hilum region

TABLE I  
PERCENTAGE OF SEEDS SWELLING IN WATER FOLLOWING VARIOUS TREATMENTS

Species	Absolute ethyl alcohol, 72 hrs.	Shaking 20 min.	Filed	Intact
Mimosoideae				
<i>Acacia aneura</i>	29	75	100	18
" <i>constricta</i>	88	20	100	9
" <i>greggii</i>	100	100	100	7
" <i>hakeoides</i>	10	70	100	0
" <i>linifolia</i>	46	6	93	2
" <i>pycnantha</i>	16	30	100	18
" <i>saligna</i>	34	39	98	14
<i>Leucaena pulverulenta</i>	14	3	100	3
<i>Prosopis velutina</i>	74	100	100	30
Papilionoideae				
<i>Cladrastis lutea</i>	10	82	100	6
" <i>amurensis</i>	26	100	100	21
<i>Cytisus scoparius</i>	22	45	99	2
<i>Melilotus alba</i>	0	86	100	1
<i>Robinia pseudo-acacia</i>	58	98	99	56
Caesalpinoideae				
<i>Cassia artemisoides</i>	57	3	100	2
" <i>leptocarpa</i>	97	37	100	10
<i>Cercidium floridum</i>	100	12	100	2
<i>Gleditsia triacanthos</i>	80	5	100	5
<i>Gymnocladus dioica</i>	92	4	100	0
<i>Parkinsonia aculeata</i>	100	8	100	2
" <i>microphylla</i>	100	90	100	10
<i>Cercis chinensis</i>	98	10	100	6

as described by Verschaffelt or is due to the leaching out of some chemical as claimed by others, seeds so treated begin swelling at the hilum region. Seeds belonging to the subfamily Mimosoideae seem to occupy a position between those of the other two subfamilies as regards their response to shaking and soaking in alcohol (Table I). Within the genus *Acacia*, for example, 88 per cent of *A. constricta* seeds took up water after soaking in alcohol while shaking had very little effect. More *A. aneura* seeds were made permeable by shaking than by soaking in alcohol while 100 per cent of *A. greggii* seeds became permeable as a result of either treatment.

Impermeable seeds of *Melilotus alba* could be made to absorb water by

plunging them in liquid nitrogen ( $-195.8^{\circ}\text{C}.$ ). Four dips of thirty seconds each into the liquid were more effective than one dip of one or five minutes' duration. One minute was allowed to elapse between the dips. If the seeds were plunged into water at room temperature before and after each dip, they gave 97 per cent germination when placed subsequently on filter paper moistened with water. Seeds dipped four times without being plunged into water gave 38 per cent germination, and untreated hard seeds failed to germinate. Liquid nitrogen treatment, even when extended to fifteen minutes' duration, had no effect on impermeable seeds of *Gleditsia tria-*

TABLE II  
PER CENT SEEDLING PRODUCTION BY JULY, 1946, FROM PLANTINGS MADE IN THE FIELD

Species	No. of seeds in planting	Planted December 4, 1945		Planted April 22, 1946	
		Intact	Filed	Intact	Filed
Mimosoideae <i>Acacia decurrens</i>	5 X 100	0	0	0	20
Papilionoideae <i>Cladrastis lutea</i> <i>Melilotus alba</i>	5 X 50 10 X 100	15 41	3 1	2 1	13 84
Caesalpinoideae <i>Cassia artemisoides</i> <i>Cercis canadensis</i> <i>Gleditsia triacanthos</i> <i>Gleditsia triacanthos</i> var. <i>inermis</i>	10 X 100 10 X 100 4 X 50 5 X 50	0 0 23 21	1 5 0 0	0 0 12 12	22 45 36 63

*canthos*. These results again pointed to inherent differences in the causes of impermeability of the two kinds of seeds.

Freezing has been found by others (1, 6) to make seed coats of *Melilotus* and alfalfa permeable.

It became of interest to determine whether weathering effects in field plantings would be confined to the Papilionoideae subfamily. Consequently seeds were planted in the field on December 4, 1945, and again on April 22, 1946. There had been periods of cold weather including some snow, previous to the December planting, but the soil was entirely free of frost, though very wet, on the planting date. The species used and the seedling production in soil in the spring and early summer of 1946 are shown in Table II. A previous experiment pictured in Figure 1 had shown us what to expect in the case of *Melilotus alba*. Forty-one per cent seedling production was secured in the spring from impermeable seeds planted in December. Only 1 per cent of the impermeable seeds planted in April produced seedlings. Filed seeds planted in April gave 84 per cent germination. Filed seeds planted in December absorbed water readily and rotted because of the

unfavorable germination temperatures so no seedlings were secured. *Cladrastis lutea* might be expected to behave in a similar manner, and although germinations were few the general effect was the same. In *Acacia*, *Cassia*, and *Cercis* no softening of the hard-coated seeds resulted from a winter in the field but in *Gleditsia* there may have been some effect.

It may well be that there are at least two different types of impermeability in the coats of seeds of the legume family and that these types are



FIGURE 1. Seeds of *Melilotus alba*. A. Planted in October (3 rows); B. filed seeds planted in May (1 row); and C. intact seeds planted in May. Photographed the following July.

indicated by their response to either shaking, or alcohol treatment. In either case, the vulnerable region of the coat seems to be near the hilum which region probably is the one affected in the softening of the coat in nature. Certain treatments effective for all types, such as concentrated sulphuric acid treatment, and mechanical abrasion, act on the whole seed surface and are not confined to any one region of the coat.

#### SUMMARY

Tests conducted to make hard coats of some leguminous seeds permeable revealed certain distinguishing characters of subfamily groups. Seeds of members of the Papilionoideae were made permeable by shaking in a glass bottle for twenty minutes, but soaking in absolute alcohol was with-

out effect. Seeds of the Caesalpinioideae, on the other hand, were made permeable by soaking in absolute alcohol for 72 hours, but, with one exception, were not affected by shaking. Seeds of Mimosoideae seemed to occupy an intermediate position, some responding to shaking and some to alcohol soaking.

Impermeable seeds of *Melilotus alba* (Papilionoideae) could be made to absorb water by plunging them in liquid nitrogen. This treatment had no effect on impermeable seeds of *Gleditsia triacanthos* (Caesalpinioideae).

The behavior of impermeable seeds after treatment with certain other chemicals and under field conditions is also reported.

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# NITROGEN AVAILABILITY IN SOILS AS MEASURED BY GROWTH RESPONSE OF RYE GRASS AND CUNNINGHAMELLA BLAKESLEEANA

M. M. McCool

It may be that the economical aspects of the use of nitrogen on pastures will change radically at some future date. Thus there may be a need for a relatively short and inexpensive method or methods for determining nitrogen availability in soils.

A study of the availability of nitrogen in 89 samples of soil which represent 14 types has been conducted. In these investigations the nitrogen availability was measured by the growth response of rye grass (*Lolium perenne* L.) to nitrogen in greenhouse and field cultures, and, in addition, the effect of nitrogen on the development of colonies of *Cunninghamella blakesleeana* was ascertained. The results derived from the use of these methods of procedure, with few exceptions, show the same trend, notably a deficiency of available nitrogen in the soils under investigation.

## MATERIALS AND METHODS

The soil samples were taken by the writer either in early October or April to the depth of plowing from soils devoted to grazing in the states of New Jersey, Pennsylvania, and New York. The immediate surface was discarded. The samples of a given soil were removed from widely separated localities. The numbers taken varied because of variations with respect to distance, highways, and weather conditions. They were brought to the laboratory, screened, mixed, and placed in the containers for plant growth studies while in the moist condition. Glazed jars of two-gallon capacity were utilized as containers in the grass grown in the greenhouse. The receptacles for the soils in the field tests consisted of wooden boxes, 13 inches in width, 18 inches in length, and 8 inches in depth. The surface soil was removed and the boxes without bottoms were so arranged that the tops were about one-half of one inch above the surface of the soil, thus preventing surface water from entering them.

The samples employed in the fungus cultures were air dried before treatment.

The basic treatment of the soil for plant growth studies consisted of 500 parts per million of superphosphate (16 per cent  $P_2O_5$ ), 50 parts per million of potassium chloride, and 1500 parts per million of calcium carbonate, and where the plans called for nitrogen, 150 parts per million of nitrate of soda. The latter was applied in solution. Two grams of seed were sown in the two-gallon jars and proportionate amounts in the other containers.

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The cultures were arranged at random in blocks on the greenhouse bench and in the field. The period of growth was 30 days for the cultures in the jars. The grass was harvested in the field trials 40 days after planting.

The plaques or containers described by Mehlich (5), in which were placed the cultures of the fungus, were arranged at random in a circular pan two feet in diameter and four inches in depth. The covered pan which revolved slowly was placed in a room maintained at 29° C. Air of the proper temperature was brought into and circulated within the room by means of an electrically driven fan. The cultures were set on absorbent cotton which was saturated with water. The cover, which fitted loosely over the pan, was also covered loosely with cotton. This arrangement resulted in similar conditions with respect to aeration and temperature. The media employed for nitrogen deficiency determinations were made up as given by Mehlich (5).

#### REVIEW OF LITERATURE

Lyon (4) conducted fertilizer tests with bulk samples of Ontario, Volusia, and Dutchess soil types which were taken from widely separated locations. He found different samples of each type to vary greatly in their need for phosphorus, and all of them evidenced a more striking need for nitrogen than for phosphorus.

Gustafson and associates (1, 2, 3, 7, 8, 9) have called attention to a general deficiency of available nitrogen for the production of grass on various soils of New York State. Owing to the unit cost of nitrogen, however, the addition of it from commercial sources is not considered to be profitable except on dairy farms which lie relatively near Metropolitan areas.

Mehlich (5) who perfected the *Cunninghamella blakesleeana* fungus method for studying the nitrogen availability of soils reports it to distinguish between soils low and those high in available nitrogen. He considers the growth of the fungus should attain a diameter of 28 to 32 millimeters in a given soil for the nitrogen availability to be sufficient to meet the requirements of field crops.

Mooers (6, p. 211) employed 129 samples of soil taken from what were termed "9 distinct soil types, 1 soil type phase, and 1 land type." The majority of the samples were collected from fertilizer test plots. The fungus method (5) enabled him to distinguish between fertilized and unfertilized plots.

#### RESULTS

The yields of grass in the greenhouse tests and the diameter of the colonies of *Cunninghamella* which developed on nine different samples of Dutchess soil are given in detail in Table I. The increase in growth of these upon the addition of nitrogen to the cultures and those from other soils

are so striking and consistent, it did not appear essential to present other than the average of results obtained (unless otherwise given the averages represent three replicates) in the remainder of the experiments. It is to be noted furthermore that the rye grass responded strikingly to the addition of nitrogen to all samples except sample No. 5 of Merrimac loam and Lords-town sample No. 2 of Table II A. The yields from different samples of a given soil type, however, varied rather widely.

The diameter of the colonies of *Cunninghamella*, with four exceptions, notably Merrimac loam and samples 6, 8, and 10 of the Weatherfield type

TABLE I  
RESULTS WITH DUTCHESS SOIL TYPE

Samples from different localities	Fresh weight of grass in grams, greenhouse						Diameter of colonies of fungus in millimeters			
	No N			+N			No N		+N	
	Blocks			Blocks						
	I	II	III	I	II	III	Repl. I	Repl. II	Repl. I	Repl. II
1	32	33	35	71	68	73	20	21	32	30
2	35	30	32	58	56	62	16	16	37	34
3	36	31	32	83	88	84	10	12	25	23
4	28	31	33	72	74	78	9	10	42	39
5	26	21	23	96	63	86	17	17	45	46
6	22	20	23	50	51	54	16	15	34	33
7	25	27	27	60	53	55	19	15	43	42
8	25	26	24	56	50	51	18	19	42	40
9	26	24	29	50	58	56	14	14	26	24

(Table II A), were significantly greater where nitrogen was included in the cultural solution than it was in those from which it was omitted. The development of the colonies varied greatly within each group of soils.

It is possible that the growth of the fungus, for example, was retarded or stimulated by some factor or factors which did not affect the growth of the grass.

Throughout the conduct of the tests with grass it was observed that nitrogen response was evident as indicated by the color and length of growth of the seedlings within ten days after germination and the lack of available nitrogen as determined by the harvest later could be predicted.

A comparison of the results derived from the use of each of the three methods with samples taken from each of four soil types for these tests may be had from an examination of the data which comprise Table III. Great response of the grass took place upon the addition of nitrogen to each of these soils in the field. In the greenhouse tests the increase in growth which resulted from the application of nitrogen was also striking. The diameter



of the colonies of *Cunninghamella* was increased significantly by adding nitrogen to the medium.

*Field tests.* The writer took advantage of an opportunity to obtain in-

TABLE II  
EFFECT OF ADDITIONS OF NITROGEN ON THE WEIGHT OF RYE GRASS TOPS,  
AND DIAMETERS OF FUNGUS COLONIES

Soil sample No.	Lackawanna				Gloucester				Weatherfield				Lordstown				Merrimac F.S.L.			
	Grass, wt. g., green-house		Fungus, diam. mm.		Grass, wt. g., green-house		Fungus, diam. mm.		Grass, wt. g., green-house		Fungus, diam., av. 2 cultures mm.		Grass, wt. g., green-house		Fungus, diam., av. 2 cultures mm.		Grass, wt. g., green-house		Fungus, diam. mm.	
	No	+	No	+	No	+	No	+	No	+	No	+	No	+	No	+	No	+	No	+
	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
1	24	46	34	42	33	87	25	41	1	6	23	34	3	10	20	30	3	9	24	27
2	54	87	30	41	56	95	10	27	1	6	24	33	10	11	26	33	4	9	25	33
3	51	100	27	40	73	102	11	29	1	6	25	34	4	10	29	36	3	12	13	28
4	30	75	10	39	42	75	15	32	3	6	26	42	7	10	23	32	6	11	26	32
5	27	72	13	35	52	107	28	41	5	8	27	41	5	10	24	35	9*	10	37	39
6	42	75	14	34	39	87	19	33	4	9	27	25	3	10	31	38				
7	34	96	20	42	26	66	18	47	5	7	24	31								
8	36	94	25	42	41	85	18	46	5	7	25	26								
9	73	123	23	29	33	64	12	31	5	10	24	33								
10					23	40			3	8	22	23								

\* Merrimac loam.

## B

Soil type	Grass, wt. g., greenhouse		Fungus, diam., mm.	
	No	+	No	+
	N	N	N	N
Mardin	11	35	26	44
Langford	27	40	31	45
Culvers	32	40	27	46
Dunkirk	19	39	23	32
Volusia	10	39	17	53
Ontario	28	36	28	34

## C

Soil sample No.	Diameters of fungus colonies, mm., average of 2 cultures					
	Mardin		Culvers		Langford	
	No	+	No	+	No	+
	N	N	N	N	N	N
1	23	48	32	47	22	26
2	19	46	23	49	19	32
3	18	44	27	36	25	42
4	25	49	30	53	28	39
5	22	53	13	31	9	22
6	27	52	24	47	12	27
7	22	40	17	46	27	39
8	19	40	29	46	16	46
9	21	30	30	52	32	42

formation relative to pasture land improvement in the spring of 1933 on the farm of A. J. McNab near Shekomiko, Dutchess County, New York. At that time the covering of the land was typical of unfertilized pastures on Dutchess soils, that is, thin stand of grass and weedy. One portion of a

pasture was treated with superphosphate at the rate of 500 pounds per acre, on another the same amount of phosphate and 300 pounds per acre of calcium cyanamid were added. The plans called for annual fertilization with the latter and with phosphate after four years. The cyanamid application was reduced to 200 pounds per acre after the fourth year. The results derived from the phosphated portion were disappointing the first and subsequent years, but where the cyanamid was applied striking results have been derived throughout the period. Excellent yields of forage were obtained, and rapid thickening of the stand of Kentucky blue grass and also an excellent stand of wild white clover appeared after the third season. Thus it was possible and practicable to thicken the stand of grass, increase the yield tremendously, and at the same time bring in wild white clover. It

TABLE III  
RESULTS FROM FIELD, GREENHOUSE, AND FUNGUS TESTS

Soil type	Field tests, fresh weight of grass in grams		Greenhouse tests, fresh weight of grass in grams, average of 3 cultures		Fungus tests, diameter of colonies of fungus in milli- meter, average of 2 cultures	
	No N	+N	No N	+N	No N	+N
Podunk	28, 32, 35	104, 95, 101	7.7	18.4	25	37
Gloucester	37, 26, 31	86, 93, 88	7.9	18.8	18	35
Dutchess	39, 49, 46	114, 107, 108	8.1	13.1	18	41
Weatherfield	33, 32, 49	103, 119, 95	8.3	17.8	22	36

would appear that there are great possibilities for pasture land improvement on soil such as this one studied by following this program, especially where conditions are such that manure and limestone cannot be added to it.

Samples also were taken from Mardin, Langford, Culvers, Dunkirk, Volusia, and Ontario soils and the response of rye grass and also *Cunninghamella* to the addition of nitrogen ascertained. According to the data which comprise Table II, B and C, each sample was deficient in available nitrogen as measured by the growth response of grass and fungus colonies when nitrogen was added.

It should also be noted that the fungus method of procedure has been employed rather extensively with samples of local soil which have been mailed to or brought to the Boyce Thompson Institute by owners of lawns and vegetable gardens, in order to obtain information relative to their nitrogen requirements for the growth of plants in question.

#### SUMMARY AND CONCLUSIONS

Three methods were employed in studying the availability of the nitrogen in 89 soil samples taken from 14 soil types. The growth of rye grass

under greenhouse and field conditions and also the response of *Cunninghamella blakesleeana* to the addition of nitrogen to the soil were ascertained. The results derived from the use of these methods of procedure with few exceptions show the same trend, notably a deficiency of available nitrogen in the soils studied. With the exceptions of one sample of Merrimac loam, one of Gloucester, and one of Lordstown, the soils responded to nitrogen in the greenhouse studies. The fungus did not respond to the addition of nitrogen to four samples, notably Merrimac loam and three of Weatherfield.

The liberal use of superphosphate on Dutchess silt loam soil type devoted to the production of grass for dairy cattle in Dutchess County was disappointing, but where calcium cyanamid was used with it the results were a thickened stand of grass, the bringing in of wild white clover, and striking increases of forage production.

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# PREPARATION OF A SERIES OF $\omega$ -(2,4-DICHLOROPHENOXY)-ALIPHATIC ACIDS AND SOME RELATED COMPOUNDS WITH A CONSIDERATION OF THEIR BIOCHEMICAL ROLE AS PLANT-GROWTH REGULATORS

MARTIN E. SYNERHOLM AND P. W. ZIMMERMAN

Recent years have seen much activity in the field of plant-growth regulators of the aryloxyaliphatic acid type. Particular attention has been paid 2,4-dichlorophenoxy-acetic acid ("2,4-D"), 2-methyl-4-chlorophenoxy-acetic acid ("Methoxone"), and  $\beta$ -naphthoxyacetic acid. The literature pertaining to members of this class of compounds has been reviewed extensively (2, 3, 9, 10, 11).

As a contribution to the steadily increasing collection of published information on this class of compounds, it was considered worth while to study a series of aryloxyaliphatic acids differing only in the number of carbon atoms in the aliphatic acid part of the molecule while retaining the aryloxy radical on the terminal ( $\omega$ ) carbon atom. Because of the pronounced physiological activity of 2,4-dichlorophenoxyacetic acid, such a series of 2,4-dichlorophenoxyaliphatic acids was decided upon. Thompson *et al.* (10) include in their list of compounds (published during the course of this work)  $\beta$ -(2,4-dichlorophenoxy)propionic acid, which they found by the corn-germination test to be practically inactive—actually, their data indicate a slight activity opposite in direction to that produced by the next lower and very active homolog, 2,4-dichlorophenoxyacetic acid. They also include  $\gamma$ -(2,4-dichlorophenoxy)butyric acid, which appeared to equal the acetic acid analog in activity in the same test. In the present work, the series is carried through the member with eight carbon atoms in the aliphatic acid moiety. For comparison of the chemical structures, the formulas for this series are shown in Table I.

Because, in the case of the free acids, it was sometimes difficult to obtain sharp-melting samples, it was believed advisable to prepare and compare the more easily purified amides. Furthermore, it was noted that, in the cases where the free acids caused burning of the plant tissue—making reliable observation of the "hormone" properties difficult—the amides did not burn within the range of concentrations studied. The amides are shown in Table II.  $\gamma$ -(*o*-Chlorophenoxy)-,  $\gamma$ -(2,4,5-trichlorophenoxy)-,  $\gamma$ -(2,4,6-trichlorophenoxy)-, and  $\gamma$ -( $\beta$ -naphthoxy)butyric acids as well as  $\beta$ -( $\beta$ -naphthoxy)propionic acid were prepared and tested for comparison with their analogs in the 2,4-dichlorophenoxy series. These are shown in Table III.

TABLE I  
 $\omega$ -(2,4-DICHLOROPHENOXY)ALIPHATIC ACIDS

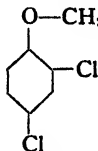
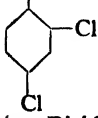
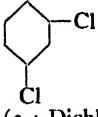
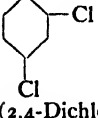
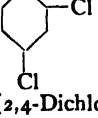
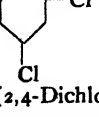
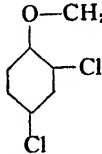
Compound*	M.p. °C. (uncorr.)	Physiological activity**	
		Cell elongation	Local injury
$\text{O}-\text{CH}_2 \cdot \text{CO}_2\text{H}$  2,4-Dichlorophenoxyacetic acid	139	0.08	50
$\text{O}-\text{CH}_2 \cdot \text{CH}_2 \cdot \text{CO}_2\text{H}$  $\beta$ -(2,4-Dichlorophenoxy)propionic acid	93	Inactive	50
$\text{O}-\text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CO}_2\text{H}$  $\gamma$ -(2,4-Dichlorophenoxy)butyric acid	117	1.0	50
$\text{O}-\text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CO}_2\text{H}$  $\delta$ -(2,4-Dichlorophenoxy)valeric acid	65	Inactive	100
$\text{O}-\text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CO}_2\text{H}$  $\epsilon$ -(2,4-Dichlorophenoxy)caproic acid	92	0.3	100
$\text{O}-\text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CO}_2\text{H}$  $\zeta$ -(2,4-Dichlorophenoxy)enanthic acid	67	Inactive	50

TABLE I (Continued)

Compound*	M.p. °C. (uncorr.)	Physiological activity**	
		Cell elongation	Local injury
$\text{O}-\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CO}_2\text{H}$  $\eta$ -(2,4-Dichlorophenoxy)caprylic acid	108-111	0.625	None

\* With the exception of the acetic acid member, these compounds have not been previously reported with proofs of their identities insofar as the authors are aware.

\*\* The figures in these columns are approximately the lowest concentrations (mg./g.) in lanolin which caused the indicated effect. When a compound is listed as inactive for cell elongation or local injury, these observations were made at the concentration of 100 mg./g. of lanolin.

PREPARATION OF COMPOUNDS<sup>1</sup>

*2,4-Dichlorophenoxyacetic acid and amide.* The preparation of this material, popularly known as "2,4-D," has been adequately described in the literature (7) and is now commercially available. The amide (m.p. 152 to 153°) was prepared by allowing an excess of thionyl chloride to react with 2,4-dichlorophenoxyacetic acid dissolved in benzene and pour-

TABLE II  
 $\omega$ -(2,4-DICHLOROPHENOXY)ALIPHATIC ACID AMIDES

Compound*	M.p. °C. (uncorr.)	Per cent chlorine†		Physiological activity**	
		Calcd.	Obs.	Cell elongation	Local injury
2,4-Dichlorophenoxyacetamide	152	—	—	0.03	None
$\beta$ -(2,4-Dichlorophenoxy)propionamide	104	30.3	30.4	Inactive	None
$\gamma$ -(2,4-Dichlorophenoxy)butyramide	104	28.6	28.3	0.3	None
$\delta$ -(2,4-Dichlorophenoxy)valeramide	97	27.1	27.2	Inactive	None
$\epsilon$ -(2,4-Dichlorophenoxy)caproamide	89	25.7	25.6	0.1	None
$\zeta$ -(2,4-Dichlorophenoxy)heptanamide	73	24.4	24.7	Inactive	None
$\eta$ -(2,4-Dichlorophenoxy)caprylamide	83-84	23.3	23.0	0.625	None

\* To the authors' knowledge with the exception of the acetamide member, these compounds are reported here for the first time.

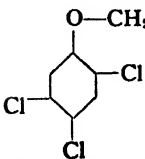
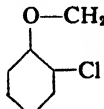
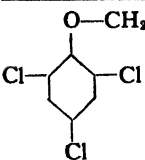
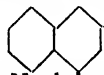

\*\* See footnote \*\*, Table I.

† The observed values were obtained by the Parr bomb-fusion method.

<sup>1</sup> The authors wish to acknowledge the assistance of Mr. Victor Cullmann, who prepared certain intermediates needed for this work.

ing the reaction mixture into an excess of 28 per cent ammonia. The amide was caused to crystallize from the benzene by the addition of an equal volume of petroleum ether (30 to 60°), and was filtered and recrystallized from a benzene-petroleum ether mixture.

TABLE III  
MISCELLANEOUS  $\omega$ -ARYLOXYALIPHATIC ACIDS

Compound	M.p. °C. (uncorr.)	Neutral equivalent		Physiological activity**	
		Calcd.	Obs.	Cell elongation	Local injury
 $\gamma$ -(2,4,5-Trichlorophenoxy)butyric acid*	112-113	283.6	284	Inactive	None
 $\gamma$ -(o-Chlorophenoxy)butyric acid*	84	214.5	217	Inactive	None
 $\gamma$ -(2,4,6-Trichlorophenoxy)butyric acid*	119-120	283.6	282	Inactive	None
 $\beta$ -( $\beta$ -Naphthoxy)propionic acid	140-141 (lit. 144-5°)	216.2	218	Inactive	None
 $\gamma$ -( $\beta$ -Naphthoxy)butyric acid*	120	230.2	229	0.15	None

\* These compounds have not been previously reported in the literature.

\*\* See footnote \*\*, Table I.

*$\beta$ -(2,4-Dichlorophenoxy)propionic acid and amide.* The method used to prepare the  $\beta$ -(2,4-dichlorophenoxy)propionic acid is an adaptation of Powell's procedure (6) for the preparation of  $\beta$ -phenoxypropionic acid involving the formation of the intermediate 3-phenoxypropanol and oxidation of this alcohol with potassium permanganate to the acid.

Five and seven-tenths grams (0.25 atom) of sodium were dissolved in 200 ml. of absolute alcohol. Forty-one grams (0.25 mole) of 2,4-dichlorophenol were then added, followed by 35 g. (0.25 mole) of trimethylene bromohydrin. The mixture was refluxed for three hours. The alcohol was removed on the steam bath after which benzene was added and the sodium bromide and any excess of 2,4-dichlorophenol removed by washing with 10 per cent aqueous sodium hydroxide solution. The benzene layer was separated, dried over sodium sulphate, and distilled. The 3-(2,4-dichlorophenoxy)propanol was collected at 155 to 160°/2 mm.; yield, 18 g. (33 per cent).

A mixture consisting of 18 g. (0.08 mole) of the 3-(2,4-dichlorophenoxy)propanol, 36 g. of magnesium sulphate (to maintain neutrality of the mixture), and 0.5 g. of sodium lauryl sulphate (as emulsifier) in 120 ml. of water was stirred vigorously while 340 ml. of a 5 per cent potassium permanganate solution were dropped in slowly over a period of about eight hours. Stirring was continued for another twelve hours or until the color of the permanganate had disappeared. Enough sodium bisulphite was added to destroy the manganese dioxide. The clear solution was filtered from some gummy material that had formed and was acidified with 50 per cent sulphuric acid. The  $\beta$ -(2,4-dichlorophenoxy)propionic acid separated first as an oil but soon solidified and was filtered and dried. After recrystallization from a benzene-petroleum ether mixture, it melted at 93°; yield, 2 g. (10 per cent). *Neut. equiv.* Calcd. for  $C_8H_7OCl_2CO_2H$ : 235.1. Found: 236.

The amide prepared by the method described for its lower homolog melted at 104 to 105°. *Anal.* Calcd. for  $C_9H_9O_2Cl_2N$ : Cl, 30.3. Found: Cl, 30.4.

*$\gamma$ -(2,4-Dichlorophenoxy)butyric acid and amide.* To a solution of sodium ethoxide prepared from 7.1 g. (0.31 atom) of sodium and 200 ml. of absolute alcohol were added 50 g. (0.31 mole) of 2,4-dichlorophenol followed by 80 g. (0.51 mole) of trimethylene chlorobromide. The mixture was refluxed for three hours. The alcohol was removed on the steam bath. Benzene was added and the mixture was extracted with dilute aqueous sodium hydroxide solution to remove the excess 2,4-dichlorophenol and the sodium bromide formed. The benzene layer was separated and distilled under diminished pressure. The yield of 3-(2,4-dichlorophenoxy)propyl chloride (b.p. 185 to 187°/34 mm.) was 58 g. (78 per cent yield).

A mixture of 20 g. (0.083 mole) of 3-(2,4-dichlorophenoxy)propyl chloride and 10 g. (0.204 mole) of sodium cyanide in 200 ml. of alcohol was refluxed four hours on the steam bath. The mixture was poured into water. The nitrile separated as an oil and was extracted with benzene. After removal of the benzene, the crude nitrile was saponified by refluxing for five hours with 10 g. of potassium hydroxide in 50 per cent alcohol. The



acid which separated on acidification of the mixture was taken up in benzene from which it was extracted with dilute sodium bicarbonate. Acidification of the aqueous extract gave the pure acid. One recrystallization from benzene-petroleum ether gave a product melting at 117 to 118°. *Neut. equiv.* Calcd. for  $C_9H_9OCl_2CO_2H$ : 249.1. Found: 253.

The amide, prepared in the usual manner, melted at 104 to 105°. *Anal.* Calcd. for  $C_{10}H_{11}O_2Cl_2N$ : Cl, 28.6. Found: Cl, 28.3.

*δ*-(2,4-Dichlorophenoxy)valeric acid and amide. To a solution of sodium ethoxide prepared from 3.6 g. (0.156 atom) of sodium and 75 ml. of absolute alcohol were added 25 g. (0.156 mole) of diethyl malonate. The 3-(2,4-dichlorophenoxy)propyl chloride [37.5 g. (0.156 mole)] was added all at once and the mixture was refluxed for five hours while being protected from atmospheric moisture with a drying tube. To the alcoholic mixture were added 50 ml. of 40 per cent sodium hydroxide solution and, after heating for about an hour on the steam bath, the mixture was poured into water and extracted with benzene to remove the excess dichlorophenoxypropyl chloride. The aqueous layer was separated and acidified with dilute sulphuric acid. The mixture was extracted with benzene. A solid benzene-insoluble fraction, presumably bis[3-(2,4-dichlorophenoxy)propyl]malonic acid, was filtered from the benzene solution of the desired product. Removal of the benzene left an oil [*Neut. equiv.* Calcd. for  $C_{10}H_{10}OCl_2(CO_2H)_2$ : 153.5. Found (for the crude material): 148] which was decarboxylated at 200° in a Wood's metal bath to yield 7 g. of the monocarboxylic acid as an oil which solidified only very slowly. The crude *δ*-(2,4-dichlorophenoxy)-valeric acid was converted by the usual procedure into its amide, m.p. 97 to 98° from benzene-petroleum ether; yield, 5 g. *Anal.* Calcd. for  $C_{11}H_{12}O_2Cl_2N$ : Cl, 27.1. Found: Cl, 27.2.

*ε*-(2,4-Dichlorophenoxy)caproic acid and amide. To a solution of 7.52 g. (0.326 atom) of sodium in 200 ml. of absolute alcohol were added 53.3 g. (0.326 mole) of 2,4-dichlorophenol. The resulting solution was added slowly with mechanical stirring to a refluxing solution of 150 g. (0.652 mole) of pentamethylene bromide in 200 ml. of absolute alcohol. After the addition, the mixture was refluxed until neutral toward moist litmus paper. The mixture was poured into water and the oil separated. The oil, after washing first with a little dilute alkali then with water, was distilled. The principal fraction, 5-(2,4-dichlorophenoxy)pentyl bromide, boiling at 177 to 179°/6 mm., weighed 63 g. (61.7 per cent). A small amount of high boiling material (b.p. 250°/8 mm.), presumably 1,5-bis(2,4-dichlorophenoxy)pentane, came over but was not identified. This material solidified on cooling and melted at 77°.

Twenty-five grams (0.080 mole) of 5-(2,4-dichlorophenoxy)pentyl bromide, 10 g. of sodium cyanide and 100 ml. of alcohol were refluxed three hours. The mixture was poured into water and extracted with benzene.

After washing with water, the benzene was removed and the nitrile distilled at 192 to 193°/5 mm. Yield, 18 g. (87 per cent).

The nitrile was converted to the corresponding amide by the Radziszewski reaction: 24 g. of the nitrile, 37 ml. of 30 per cent hydrogen peroxide, 3.7 ml. of 6 N sodium hydroxide, and 50 ml. of 95 per cent alcohol were warmed at about 50° for four hours. The mixture was poured into water and the crystalline material filtered and recrystallized (m.p. 89 to 90°) from hot dilute alcohol. Yield, 15 g. (58.5 per cent). *Anal.* Calcd. for  $C_{12}H_{16}O_2Cl_2N$ : Cl, 25.7. Found: Cl, 25.6.

On acidification, the filtrate yielded about 5 g. of the corresponding  $\epsilon$ -(2,4-dichlorophenoxy)caproic acid, m.p. 92°, which gave by the usual method an amide identical with that obtained directly from the nitrile.

$\zeta$ -(2,4-Dichlorophenoxy)enanthic acid and amide. The method used in the preparation of  $\delta$ -(2,4-dichlorophenoxy)valeric acid was used in this case; starting with 2.6 g. (0.113 atom) of sodium, 18 g. (0.113 mole) of diethyl malonate and 35 g. (0.112 mole) of 5-(2,4-dichlorophenoxy)pentyl bromide, 11 g. of crude  $\zeta$ -(2,4-dichlorophenoxy)enanthic acid (oil) was obtained after the decarboxylation of the initial dibasic acid (oil) formed in the reaction.

The oily decarboxylation product was not purified but was converted to a crystalline amide, m.p. 73 to 74°. *Anal.* Calcd. for  $C_{13}H_{17}O_2Cl_2N$ : Cl, 24.4. Found: Cl, 24.7.

A small sample of the crude  $\zeta$ -(2,4-dichlorophenoxy)enanthic acid crystallized after long standing and melted at 67°.

$\eta$ -(2,4-Dichlorophenoxy)caprylic acid and amide. The method used in the preparation of the valeric and enanthic acid members of the series was used in this case. Four and six-tenths grams (0.2 atom) of sodium were dissolved in 150 ml. of absolute alcohol. To this solution of the sodium ethoxide were added 32 g. (0.2 mole) of ethyl malonate followed by 33 g. (0.135 mole) of hexamethylene dibromide. The free dibasic acid obtained solidified on standing and weighed (crude) 22 g. Fifteen grams of the dibasic acid were decarboxylated at about 200° in a Wood's metal bath and recrystallized from benzene. Even after repeated recrystallizations, the  $\eta$ -(2,4-dichlorophenoxy)caprylic acid so obtained did not appear pure (m.p. 108 to 111°). The amide, prepared in the usual way, was easily obtained pure by recrystallization from benzene-petroleum ether (30 to 60°) and melted at 83 to 84°. *Anal.* Calcd. for  $C_{14}H_{19}O_2Cl_2N$ : Cl, 23.3. Found: Cl, 23.0.

$\gamma$ -(*o*-Chlorophenoxy)butyric acid,  $\gamma$ -(2,4,5-trichlorophenoxy)butyric acid,  $\gamma$ -(2,4,6-trichlorophenoxy)butyric acid, and  $\gamma$ -( $\beta$ -naphthoxy)butyric acid. These compounds were all prepared in the usual way from the appropriate phenol and trimethylene chlorobromide, followed by reaction of the chloride with potassium or sodium cyanide and subsequent hydrolysis of

the nitrile to the acid. The details are those described in the preparation of  $\gamma$ -(2,4-dichlorophenoxy)butyric acid. These aryloxy acids are reported with their melting points and physiological activities in Table III.

*$\beta$ -( $\beta$ -Naphthoxy)propionic acid.* Ten grams (0.093 mole) of  $\beta$ -chloropropionic acid, 13 g. (0.091 mole) of  $\beta$ -naphthol, and 11 g. of potassium hydroxide in 100 ml. of water were heated at 100° overnight. The mixture was poured into water, acidified, and extracted with benzene. The benzene solution was extracted with aqueous sodium bicarbonate. The bicarbonate extract was acidified and the resulting precipitate collected by filtration. The solid, after recrystallization from benzene, weighed 1.5 g. and melted at 140 to 141°. *Neut. equiv.* Calcd. for  $C_{12}H_{11}OCO_2H$ : 216.2. Found: 218.

#### BIOLOGICAL ASSAY

The solutions in lanolin were applied to the upper side of the petiole of a leaf of a young tomato (*Lycopersicon esculentum* Mill.) plant and to the side of the adjacent stem according to the published procedure (7, 12). After 24 hours, the lowest concentration showing epinastic response was noted. These figures are shown in Tables I, II, and III in the column for "Cell elongation." In the higher concentrations, some of the chemicals caused severe local injury (burning) of the plant tissue; in these cases, the petiole lost its turgid character allowing the leaf to hang. None of the amides appeared to injure the plant at 100 mg./g. concentration or less. The figures representing the lowest concentration studied and causing local injury are shown in the tables. The compounds are listed as inactive if, at a concentration of 100 mg./g. of lanolin, there is no epinastic response. Likewise, the compounds are recorded as showing no local injury if, at 100 mg./g. of lanolin, there is no local burning of the plant tissue.

#### DISCUSSION

The two series of  $\omega$ -2,4-dichlorophenoxy compounds (acids and amides) tested vary progressively in length of carbon chain from 2,4-dichlorophenoxyacetic acid, the simplest member of the type, to  $\eta$ -(2,4-dichlorophenoxy)caprylic acid with eight carbon atoms in the carboxylic acid part of the molecule. There is not a progressive gradation of the growth promoting activity in the series. The acetic acid derivative is very active, having a threshold value (lowest concentration of the chemical in lanolin showing activity) in the neighborhood of 0.08 mg./g. of lanolin. The next higher homolog,  $\beta$ -(2,4-dichlorophenoxy)propionic acid, having an *odd* number of carbon atoms in the aliphatic acid part of the molecule is inactive for epinasty even at a concentration of 100 mg./g. of lanolin. This acid, however, does injure the tissue at the point of application. Proceeding to the next higher member of the series,  $\gamma$ -(2,4-dichlorophenoxy)butyric acid

with an *even* number of carbon atoms, it was observed that this member again is active with a threshold value of about 1.0 mg./g. The valeric acid, the next higher member of the series having an *odd*-numbered carbon chain, is again inactive at 100 mg./g. of lanolin but burns the plant at the point of application. The caproic acid, with an *even* number of carbon atoms, is active at about 0.3 mg./g. of lanolin, but the next higher homolog with an *odd*-numbered carbon chain,  $\zeta$ -(2,4-dichlorophenoxy)enanthic acid, was found to be inactive for cell elongation at 100 mg./g. of lanolin, though it showed severe burning of the tissue. Finally,  $\eta$ -(2,4-dichlorophenoxy)caprylic acid, an *even*-numbered carbon chain acid, is active at 0.625 mg./g. of lanolin.

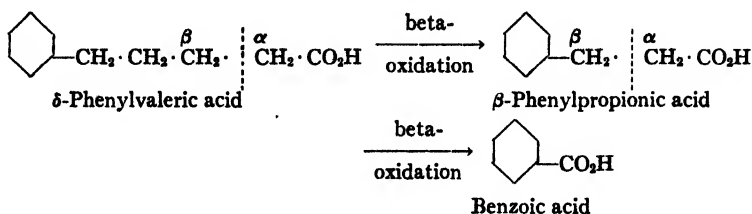
The amides in this series showed activity for cell elongation in each case in which the free acid appeared to be active. In general, the threshold values for the amides were somewhat lower than the corresponding values for the free acids. In no instance did an amide cause burning of the plant tissue at the highest concentration studied (100 mg./g.) whereas most of the acids burned at 50 mg./g. and all burned at 100 mg./g. of lanolin.

The periodicity in the activity for cell elongation of members of this homologous series of  $\omega$ -(2,4-dichlorophenoxy)aliphatic acids cannot be explained by any gradual increase in molecular weight as the number of carbon atoms increases progressively up the series. It is pertinent to suggest, however, that the members of the series higher than 2,4-dichlorophenoxyacetic acid are inactive *per se* but are converted by the plant either to the active acetic acid homolog or, in other cases, to an inactive metabolic product. That the acids are oxidized by the plant is a reasonable assumption to make in view of the role of fatty acids in other biological systems. If we should assume oxidation to occur by removal of one carbon atom at a time, it seems inevitable that all members of the series would ultimately pass through the 2,4-dichlorophenoxyacetic acid stage in the process and thus be active. If oxidation occurs by removal of two carbons in each oxidative step, only those members possessing *even*-numbered carbon chain acids could give rise to the active acetic acid member of the series. If this is indeed the case, then the situation fits into the picture which is known to exist in the animal metabolism of the fatty acids. These are known,<sup>2</sup> with few exceptions, to be oxidized at the beta carbon atom to the acid with two (or a multiple of two) fewer carbon atoms.

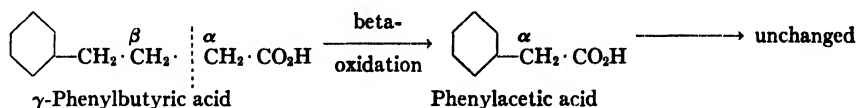
Thus, Knoop showed that  $\beta$ -phenylpropionic acid and  $\delta$ -phenylvaleric acid, when fed to dogs, are eliminated in the urine as benzoic acid in the

<sup>2</sup> For a discussion of the subject of oxidation of fatty acids in the animal organism, the reader is referred to Bodansky's "Introduction to physiological chemistry" (1, pp. 358-363) where the findings of Knoop and others are reviewed.

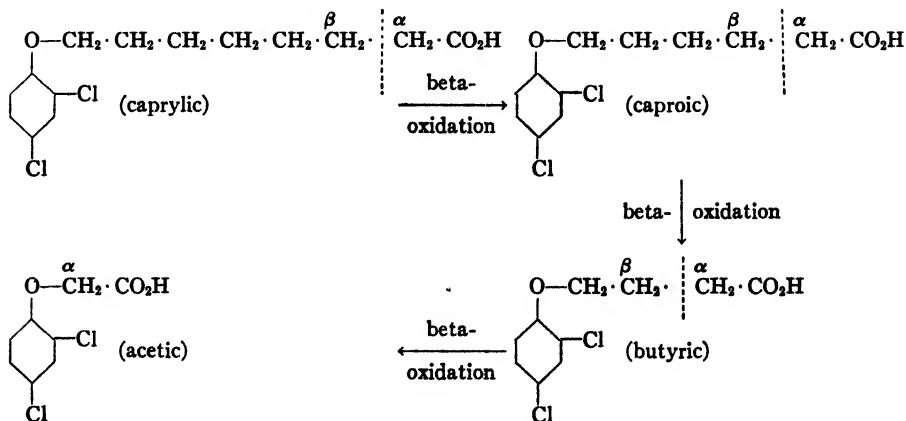
form of its amide (hippuric acid) with the amino acid glycine. The process is represented as follows:



Likewise  $\gamma$ -phenylbutyric acid gives rise to phenylacetic acid as its amide (phenaceturic acid) with glycine. Phenylacetic acid when administered is not oxidized further by the organism but is eliminated essentially unchanged as phenaceturic acid, there being no beta carbon atom as a point of oxidative attack:

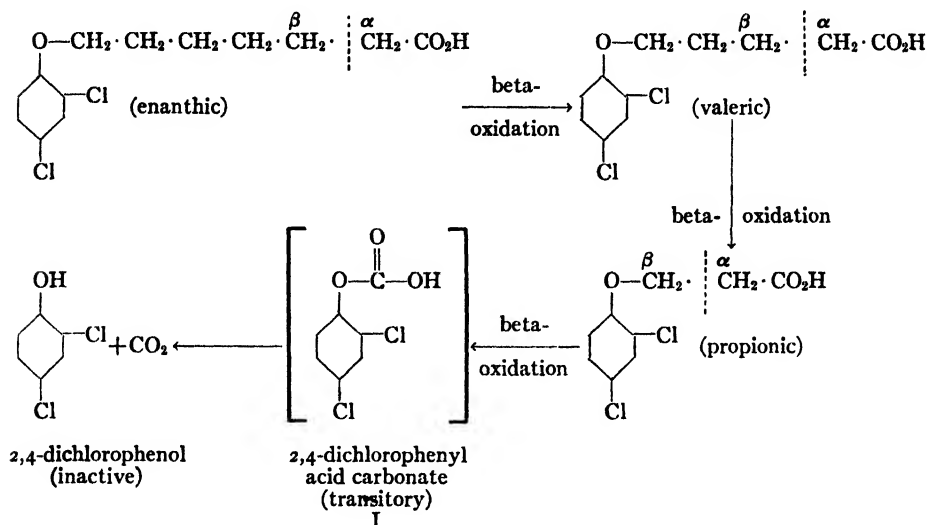


The evidence reported in this paper suggests the conclusion that the  $\omega$ -(2,4-dichlorophenoxy)aliphatic acids in their role as growth regulators are similarly attacked by the oxidase systems of the plant at the beta carbon atom to form ultimately the 2,4-dichlorophenoxy acid with two (or a multiple of two) fewer carbon atoms. Thus, the butyric, caproic, and caprylic acids (or amides), each containing an *even*-numbered carbon chain, may give rise to the acetic acid homolog, known to be a powerful growth stimulant:

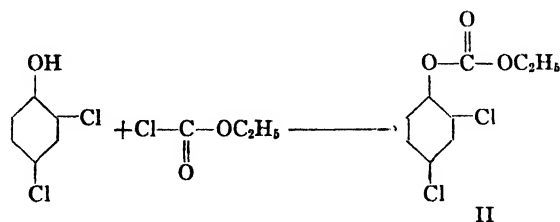


The propionic, valeric, and enanthic acids on beta-oxidation could not

result in the active acetic acid; but, as the following diagram indicates, would be converted ultimately to a half ester I of 2,4-dichlorophenol with carbonic acid, an unstable arrangement, which could only decompose to 2,4-dichlorophenol (inactive as growth stimulant) and carbon dioxide:



The hypothetical compound I, while not capable of existence *per se*, is stable as its ethyl ester II, ethyl 2,4-dichlorophenylcarbonate. This material may be prepared by the action of ethyl chlorocarbonate on 2,4-dichlorophenol:



It is reported by Thompson *et al.* (10) to be practically inactive in the kidney bean single droplet test.

It seems reasonably safe to predict, on the basis of these results, that higher members of the  $\omega$ -(2,4-dichlorophenoxy)aliphatic acid series will be physiologically active or inactive depending upon whether they contain respectively an *even* or *odd* number of carbon atoms in the aliphatic acid portion of the molecule. This generalization is necessarily subject in the case of the very high members of the series to the limitations due to the effect on the physical properties of molecular weight.

That it is impossible to extend the generalization to include all growth regulators of the aryloxyaliphatic acid type is made evident by examination of Table III.  $\gamma$ -(2,4,5-Trichlorophenoxy)butyric acid appears to be inactive even at 100 mg./g. of lanolin.  $\gamma$ -(*o*-Chlorophenoxy)butyric acid is also inactive. The acetic acid homolog of each of these compounds is a very active growth regulator. The compounds differ from their analogs in the  $\gamma$ -2,4-dichlorophenoxyaliphatic acid series only in the number of chlorine atoms in the aromatic nucleus. The shape or size or both properties of the molecule are probably important in ascribing physiological activity to the molecule.  $\gamma$ -(2,4,6-Trichlorophenoxy)butyric acid is also inactive; but this was not unexpected in view of the fact that its acetic acid homolog is also inactive for cell elongation.

$\gamma$ -( $\beta$ -Naphthoxy)butyric acid was found to be very active. Its lower homolog with one less carbon,  $\beta$ -( $\beta$ -naphthoxy)propionic acid, is inactive at 100 mg./g. of lanolin. Thus, with the known activity of the simplest member of this series,  $\beta$ -naphthoxyacetic acid, a periodicity within this series is established for the first three members.

It is of some interest to note the melting point relationships of the 2,4-dichlorophenoxy acids described. Each *odd*-numbered carbon chain acid melts lower than the next *lower even*-numbered carbon homolog. This periodicity in melting points recalls a similar relationship in some other homologous series of organic compounds—particularly the straight chain fatty acids. The melting points are probably themselves not related to physiological activity except as they are affected by molecular size, shape, and symmetry, which properties probably do influence the physiological activity of the compound. In connection with this observation, it is worth while recalling that ordinary cinnamic acid (*trans*) is inactive for cell elongation in the pea test (Haagen Smit and Went, 4), while its geometrical isomer, allocinnamic acid (*cis*) is active. Thimann and Bonner (9) in a review of plant-growth hormones in 1938 suggested that this difference in activity may be explained by the distances of the carboxyl groups from the rings in the two isomers. Kögl and Kostermans (5) had reported activities for some  $\omega$ -(3-indole)aliphatic acids which were indicative of an alternating carbon atom effect in this series. Thimann and Bonner in their review suggested that the factors influencing the activity in the two cinnamic acids may be responsible for the alternating activity in the indole series. It is not easy to accept this explanation based upon the geometry of the molecule for those members of a series which contain a very long saturated acid side chain, as do 2,4-dichlorophenoxyanthic and caprylic acids. There is no justification for assuming that, as the length of chain increases, the carboxyl group returns periodically to a position relative to the ring which is favorable for activity.

The role of the  $\omega$ -(2,4-dichlorophenoxy)aliphatic acids in the metabo-

lism of the plant is not clear. Proof that the higher members of the series of  $\omega$ -(2,4-dichlorophenoxy)aliphatic acids or the analogous  $\beta$ -naphthoxy acids are converted in the plant to the simplest member of each series—the acetic acid homolog—awaits isolation of this material from the plant which has been treated with higher members of these series. Likewise, that these acids are converted to the corresponding phenol could be unequivocally proved only by identifying this material from plants treated with them. It seems certain that, whatever the nature of the conversions of these materials, or of their intrinsic roles, a great deal more information must be obtained concerning the enzyme systems involved.

#### SUMMARY

Homologous series of  $\omega$ -(2,4-dichlorophenoxy)aliphatic acids and their amides from the simplest member, 2,4-dichlorophenoxyacetic acid through  $\eta$ -(2,4-dichlorophenoxy)caprylic acid, have been prepared and studied for possible physiological effects on growing tomato (*Lycopersicon esculentum* Mill.) plants.

The results show that in this series, only those members (acids or amides) which possess an even number of carbon atoms in the aliphatic acid portions of their molecules are active as growth regulators.

In this series, only the free acids exhibit local injury at a concentration of 100 mg./g. of lanolin. The amides do not injure at this level of concentration.

This periodicity in physiological activity is discussed from the biochemical standpoint. An extension of Knoop's theory of beta-oxidation to the biochemical role of plant-growth regulators in plants is suggested.

The periodicity of the melting points of the acids of this series is pointed out. The possibility that the regular recurrence of physiological activity may be connected to the melting point relationship through the chemical structure of the compounds is proposed.

An alternation in the physiological activity of the first three members of the  $\beta$ -naphthoxyaliphatic acid series similar to that observed in the  $\omega$ -(2,4-dichlorophenoxy)aliphatic acid series has been demonstrated.

The periodicity in activity of members of certain series of aryloxyaliphatic acids is considered in the light of a proposed explanation for a similar but much less pronounced alternate carbon effect in the 3-indole series.

About 20 organic compounds of the  $\omega$ -aryloxyaliphatic acid type, most of which are new, were examined during this investigation.

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## CHANGES IN OXYGEN, CARBON DIOXIDE, AND PRESSURE CAUSED BY PLANT TISSUE IN A CLOSED SPACE

F. E. DENNY

The occurrence of pressure changes within a container in which plant organs are respiring was reported by Harvey and Rygg (3). They found that the amount and direction of the pressure varied with different tissues. Commonly, there was first a reduction in pressure to a minimum, which occurred at the time of the exhaustion of the oxygen in the confined air, and thereafter there was a rise to positive pressures. With some of the tissues there was a preliminary rise in pressure, followed by a fall to negative values, then later to the production of a positive pressure. In a footnote in a later paper (5) they suggest that the reduced pressures observed probably are accounted for, at least in large part, by the absorption of the evolved  $\text{CO}_2$  in the fruit juice.

This paper deals with only the early stage of this process, that in which there is still remaining a good supply of oxygen in the air surrounding the tissue. Under these conditions, with all of the plants so far tested, the effect of the tissue has been to cause a reduction in pressure. By determining the amount of air needed to equalize this reduced pressure, and at the same time by estimating the amount of  $\text{CO}_2$  gained by the tissue, a comparison could be made between these two values, showing to what extent the reduced pressure accounted for the absorption of  $\text{CO}_2$  by the tissue.

Preliminary experiments with turnip roots indicated that the pressure change accounted for about 80 to 85 per cent of the gain in  $\text{CO}_2$  in the tissue. The scope of the experiments was then broadened to take into account not only the amount of reduced pressure and the gain in  $\text{CO}_2$  in the tissue, but also the change in the  $\text{O}_2$  content of the tissue, and the change in the  $\text{O}_2$  and  $\text{CO}_2$  content of the air surrounding the tissue. Tests were carried out with fruits of tomato and apple, roots of turnip and sweet-potato, and tubers of potato.

When the volume change caused by the reduced pressure was compared with the volume represented by the gain in  $\text{CO}_2$  in the tissue, the proportion which the reduced pressure bore to the  $\text{CO}_2$  gain varied with the tissue in the test. With turnip and potato about 85 per cent of the  $\text{CO}_2$  gain could be accounted for by the reduction in pressure, but with tomato and sweet-potato the proportion was less than 60 per cent, and with apple it was less than 50 per cent.

The tissues with which these low proportions were obtained are those which gave respiration quotients ( $\text{CO}_2 \div \text{O}_2$ ) higher than 1.00. This led to a

recognition of the fact that only the  $\text{CO}_2$  formed by the utilization of atmospheric  $\text{O}_2$  can bring about reduction in pressure when the  $\text{CO}_2$  is absorbed or enters into combinations with tissue constituents. The excess volume of  $\text{CO}_2$  over the volume of  $\text{O}_2$  consumed is not produced by a union with atmospheric  $\text{O}_2$  but arises directly from the molecule of the constituent entering into the respiration. This excess  $\text{CO}_2$  could increase the pressure or volume if it was not absorbed or re-combined, but it could not decrease the pressure or volume even if this occurred. It could then only restore the volume or pressure to its original condition before this excess  $\text{CO}_2$  was liberated.

Therefore, the data were reconsidered from this point of view, and the proportions computed after the amount of the excess of  $\text{CO}_2$  produced over  $\text{O}_2$  consumed had been deducted from the amount of the gain in  $\text{CO}_2$  in the tissue. Less difference was then found among the various tissues; in fact in view of the experimental errors involved it can be stated that their response was approximately the same. The proportion which the reduced pressure bore to the gain in the tissue  $\text{CO}_2$ , after correction for the excess  $\text{CO}_2$  not due to an oxidation involving atmospheric  $\text{O}_2$ , was about 75 to 85 per cent. As for the remaining 15 to 25 per cent, it is suggested that it represents  $\text{CO}_2$  remaining as a gas in the tissue not yet dissolved or combined at the end of the experiment.

#### MATERIAL AND METHODS

The plant material was obtained from the Institute garden and orchard. The organs used were: roots of turnip (*Brassica rapa* L.) var. Purple Top White Globe; roots of sweet-potato (*Ipomoea batatas* Lam.) var. Porto Rico; fruits of apple (*Pyrus malus* L.) var. Stayman Winesap; fruits of tomato (*Lycopersicon esculentum* Mill.) var. Valiant; tubers of potato (*Solanum tuberosum* L.) var. Irish Cobbler. The tissues were placed in glass desiccators of approximately 6-liter capacity equipped with glass tubes for the removal of samples of air and for the measurement of the reduced pressure within the container. All experiments were carried out in a constant temperature room at 20° C. After the desiccators were closed they were immersed in vessels of water to guard against leakage, and for further provision for temperature control. Gas samples were analyzed for  $\text{O}_2$  and  $\text{CO}_2$  by the Orsat method using a 100 cc. burette graduated to 0.2 cc. Oxygen was absorbed in pyrogallol, and  $\text{CO}_2$  in KOH.

The apparatus used for measuring the reduced pressure inside the container at the end of an experimental period is shown in Figure 1. With the desiccator still immersed in the vessel of water, with tube A open to the air, tube B is connected to the desiccator (outlet clamp from desiccator still closed). Water is then poured into the burette until it starts flowing into the

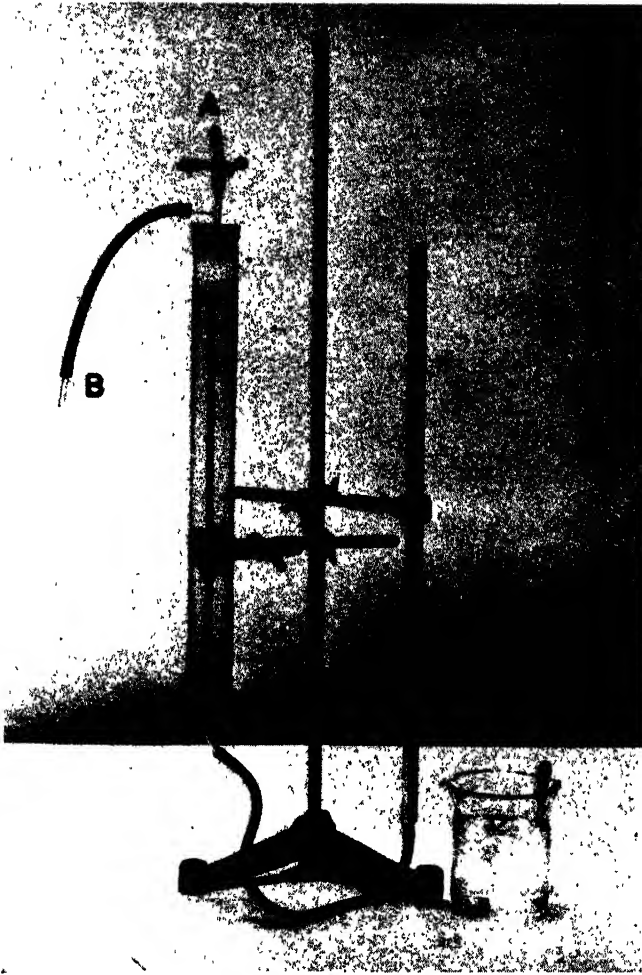


FIGURE 1. Apparatus for measuring the reduced pressure produced in a closed space by the action of plant tissue in it. With clamp A open to the air water is poured into the burette until it reaches the tip of the inner glass tubing and a note is made of the burette reading when equilibrium is reached. The beaker of water with the dropping pipette is weighed. Tube B is connected to the desiccator containing the tissue. Then clamp A is closed and the clamp leading from the desiccator to tube B is cautiously opened. As the water level falls in the burette, water is added from the beaker. When the pressure equilibrium is established (the final stage by the use of the dropping pipette) the beaker is weighed, and the difference between the two weights is a measure of the reduction in volume in the desiccator. This is the over-all effect due to changes caused both by the action of plant tissue and by barometric pressure. Correction for the amount of change in volume due to change in barometric pressure gives the net effect due to tissue action only.

large tube<sup>1</sup> (approx. 50 cm.  $\times$  5 cm.). With the water level at the tip of the inner tube the burette reading is noted. The beaker of water with the attached dropping pipette is weighed. Clamp A is then closed and the clamp leading to the desiccator is cautiously opened. As the water level in the burette falls, water is poured into it from the beaker. Finally the connection to the desiccator is made completely open, and the water in the burette is adjusted to the original mark, the last few drops being added with the pipette. When it is certain that equilibrium has been reached, the beaker and pipette are again weighed and the difference in the two weights shows the reduction in the volume of the air in the desiccator. This is the over-all volume reduction due to the combined action of the tissue on the air volume, and to the change in volume due to any change in the barometer. The change in volume due to the barometric change is computed and is either added to or subtracted from the over-all reading according to whether the barometric change has been such as to increase or decrease the volume of air when contact with external air pressure is made at the end of a test period. In experiments in which samples of the air in the desiccator are taken at the end of a test such samples are removed first, and the amount noted. This amount is then subtracted from the over-all reading of the reduced pressure.

The method of estimating the CO<sub>2</sub> content of the tissue was described in a previous article (1). The sample of tissue was disintegrated in an alkali solution in a Waring blender, and after transfer to an apparatus fitted with inlet and outlet tubes for maintaining an air current; it was acidified<sup>2</sup> and the CO<sub>2</sub> was precipitated in a solution of barium hydroxide in a series of Van Slyke-Cullen tubes. A back-titration in comparison with a blank showed the amount of CO<sub>2</sub> in the tissue. The difference between the CO<sub>2</sub> content of the sample at the start and at the end of a test showed the gain in CO<sub>2</sub> in the tissue during the interval.

The O<sub>2</sub> contents of the tissue at the start and at the end of a test were determined in a few of the experiments. The apparatus used and the procedure employed are described in a previous paper (1). Briefly, the method consisted in immersing the tissue in saturated NaCl solution, removing the

<sup>1</sup> This tube when calibrated to read volumes of water gives good results as an oxygen-supply tube in the method of determining respiration first described by Magness and Diehl (4) and later improved by Haller and Rose (2) and by Whiteman and Schomer (7). For this purpose the t-tube at the top is removed and is replaced by capillary tubing leading to the desiccator containing the respiring tissue. The inner glass tube is connected at the bottom by rubber tubing as a siphon to a constant water level. The upper tip of the inner tube is adjusted to the height of the water level.

<sup>2</sup> Note error in the previous paper in the directions for the preparation of the sulphuric acid solution to be used for acidification. In CONTRIB. BOYCE THOMPSON INST., Vol. 14, No. 4, p. 262, line 32 should read "33 cc." instead of "333 cc."

internal gas by a vacuum, transferring the extracted gas to a burette, and estimating the O<sub>2</sub> by absorption in pyrogallol.

## RESULTS

### RELATION OF REDUCED PRESSURE TO GAIN IN CO<sub>2</sub> IN TISSUE

#### *Without Regard to the Respiration Quotient*

The first tests (which later proved to be merely preliminary) were carried out with turnip roots, and the results are shown in Table I. The re-

TABLE I  
COMPARISON OF THE REDUCED PRESSURE IN CONTAINER WITH  
THE INCREASE IN CO<sub>2</sub> IN THE TISSUE  
PRELIMINARY TEST WITH TURNIP ROOTS

Lot No.	Wt. of tissue	Hrs.	Cc. CO <sub>2</sub> gain in tissue	Cc. reduced pressure inside container	% of the CO <sub>2</sub> gain in tissue accounted for by the reduced pressure
A	1027	16	55	48	87
	1024	67 5	176	153	87
B	1000	17 5	72	73	101
	1000	17 5	74	57	77
	1000	17 5	77	62	81
	1000	17 5	69	59	86
C	1000	65	200	139	69
	1000	65	201	149	74
	1000	65	193	168	87
	1000	65	193	145	75

Note: All gas volumes at 0° C. and 760 mm. Hg. press.

duced pressures found in the containers at the end of each experimental period are shown in column 5, and the corresponding amounts of gain in CO<sub>2</sub> in the tissue are given in column 4. The right hand column shows the extent to which the gain in CO<sub>2</sub> in the tissue could be accounted for by the amount of reduced pressure in the container. The reduced pressure values were about 80 to 85 per cent of the gains in CO<sub>2</sub> in the tissue.

Since it appeared that a more complete analysis of the factors involved would be required, the procedure was changed to include measurements of the CO<sub>2</sub> in the air surrounding the tissue, and of the O<sub>2</sub> in both the tissue and the air.

The results are shown in Table II; and since these showed that the change in the O<sub>2</sub> content of the tissue was small, perhaps negligible, at least for the purpose of this study, the procedure was again changed by omitting the analysis of the tissue for the O<sub>2</sub> content. A considerable saving

TABLE II  
CHANGES IN O<sub>2</sub> AND CO<sub>2</sub> IN THE TISSUES AND SURROUNDING AIR, AND  
CHANGES IN PRESSURE INSIDE THE CONTAINER

Tissue and weight	Hrs.	Cc. O <sub>2</sub> loss		Cc. CO <sub>2</sub> gain			Cc. reduced pressure inside container	% of the CO <sub>2</sub> gain in tissue accounted for by reduced pressure		R.Q.
				In air	In tissue			100× Cols. 8 ÷ 6	100× Cols. 8 ÷ 7	
		From air	From tissue		As analyzed	After corr. for excess CO <sub>2</sub>				
Tomato, 1402 g.	16 24	421 557	-0.6* -0.1	367 524	86 125	53 33	43 39	50 31	81 118	1.08 1.16
Tomato, 1420 g.	16 20.5	405 504	3.7 2.9	330 420	77 112	77 87	78 72	101 64	101 83	1.00 1.05
Tomato, 1430 g.	16 20.5	371 457	1.8 2.9	353 406	67 91	20 54	31 38	46 42	155 70	1.13 1.08
Turnip, 1500 g.	16 26	375 601	3.7 -2.4	263 466	127 178	116 133	88 175	60 98	76 132	1.03 1.08
Turnip, 1000 g.	22.5	655	5.8	517	153	144	117	76	81	1.01
Turnip, 3996 g.	18	1872	23.2	1256	561	561	438	78	78	0.96
Apple, 1250 g.	16 43.5	119 288	5.7 1.8	97 224	28 64	28 64	13 37	46 58	46 58	1.00 0.99
Sweet-potato, 987 g.	15.5	284	-0.6	207	78	76	43	55	57	1.01

Note: All gas volumes at 0° C. and 760 mm. Hg. press.

\* The negative entries in column indicate computed gains in O<sub>2</sub> content of tissues, rather than losses from it.

of time was made thereby, and it seemed better to sacrifice this information in order to increase the number of tests that could be made. The results by this procedure are shown in Table III.

Attention is first directed to the values in columns 6, 8, and 9 in Table II, and columns 4, 6, and 7 in Table III, which show the reduced pressures in the containers, the gains in the amount of CO<sub>2</sub> in the tissue during the interval, and the proportion which the reduced pressures bore to the gain in tissue CO<sub>2</sub>. The averages of the values in column 9, Table II, and column 7, Table III, are as follows:

Potato	87
Turnip	80
Tomato	57
Sweet-potato	56
Apple	48

TABLE III  
CHANGES IN O<sub>2</sub>, CO<sub>2</sub>, AND PRESSURE INSIDE OF THE CONTAINER,  
AND CHANGES IN CO<sub>2</sub> IN THE TISSUE.

Tissue, weight, and hours	Cc. O <sub>2</sub> loss from air	Cc. CO <sub>2</sub> gain			Cc. reduced pressure inside con- tainer	% of the CO <sub>2</sub> gain in tissue accounted for by reduced pressure		R.Q.
		In air	In tissue			100× Cols. 6 ÷ 4	100× Cols. 6 ÷ 5	
			As ana- lyzed	After corr. for excess CO <sub>2</sub>				
Sweet-potato, 706 g., 24 hrs.	365	322	90	43	51	57	110	1.13
	377	326	87	51	52	60	102	1.10
	347	269	79	78	60	76	77	1.00
Sweet-potato, 700 g., 24 hrs.	450	382	115	68	54	47	79	1.10
	440	367	126	73	62	49	85	1.12
	518	445	148	73	64	43	88	1.14
	500	427	147	73	49	33	67	1.15
Sweet-potato, 762 g., 21.5 hrs.	312	255	68	57	56	82	98	1.04
	465	390	122	75	76	62	101	1.10
	515	429	117	86	85	73	90	1.06
	476	424	122	52	46	38	80	1.15
Apple, 1500 g., 24 hrs.	220	188	80	32	36	45	112	1.22
	216	181	79	35	44	56	126	1.20
	196	162	72	34	26	36	77	1.19
	232	183	85	40	40	47	82	1.16
Potato, 1000 g., 72 hrs.	220	158	51	51	51	100	100	0.95
	221	154	59	59	44	75	75	0.96
	223	165	62	58	44	71	76	1.02
	214	149	44	44	45	102	102	0.90

Note: All gas volumes at 0° C. and 760 mm. Hg. press.

It will be noted that the tissues giving the low percentage values, apple, sweet-potato, and tomato, are the ones with high respiration quotients (CO<sub>2</sub> ÷ O<sub>2</sub>), as shown in the right-hand column in Tables II and III. The average R. Q. values for the different tissues (in the same order, for comparison with the list of tissues just given) are.

Potato	0.96
Turnip	1.02
Tomato	1.08
Sweet-potato	1.09
Apple	1.12

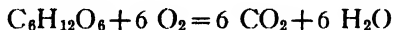
The correlation (negative) between the respiration quotients and the percentages of the gain in tissue CO<sub>2</sub> accounted for by the reduced pressure is high. This indicated a need of determining the relation of the respiration quotient to the reduction in pressure resulting from the absorption of respired CO<sub>2</sub> by plant tissue.



*After Correction for Excess CO<sub>2</sub> in Tissue*

If a given volume of O<sub>2</sub> in a closed space is converted into an equal volume of CO<sub>2</sub>, and if this CO<sub>2</sub> is dissolved or combined in some way to reduce its volume to zero, the reduction in volume in the container is equal to the volume of O<sub>2</sub> that was used up in the oxidation.

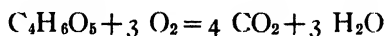
Thus, if dextrose is respired completely to CO<sub>2</sub> and H<sub>2</sub>O, the volume relationships of O<sub>2</sub> and CO<sub>2</sub> are indicated by the following equation:



$$\text{R. Q.} = 6 \div 6 = 1.00$$

That is, 6 volumes of atmospheric O<sub>2</sub> give rise to 6 volumes of CO<sub>2</sub>, and if the CO<sub>2</sub> so formed becomes dissolved or combined, the reduction in volume would be equal to the 6 volumes of O<sub>2</sub> consumed.

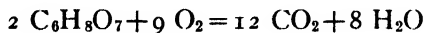
But if a substrate such as malic acid is oxidized completely to CO<sub>2</sub> and H<sub>2</sub>O, the volume relationships of O<sub>2</sub> and CO<sub>2</sub> are as follows:



$$\text{R. Q.} = 4 \div 3 = 1.33$$

That is, 3 volumes of O<sub>2</sub> give rise to 4 volumes of CO<sub>2</sub>. Notice that one of the volumes of CO<sub>2</sub> was not formed as the result of combining with atmospheric O<sub>2</sub> directly; this volume of CO<sub>2</sub> was derived from the malic acid molecule itself. The malic acid molecule was already partially oxidized, its O<sub>2</sub> content being high because of a previous partial oxidation. This extra volume of CO<sub>2</sub> would not bring about any reduction in volume in a closed space such as is dealt with in these experiments, although it could increase the volume (or pressure) if it was not dissolved or recombined. It, thus, seems clear that only CO<sub>2</sub> produced by combination with atmospheric O<sub>2</sub> can cause reduction in volume or pressure in a closed space because of being subsequently dissolved or combined in such way as to reduce its volume to zero.

A similar argument can be made in the case of the complete oxidation of citric acid, which furnishes an equation as follows:



$$\text{R. Q.} = 12 \div 9 = 1.33$$

That is, 9 volumes of O<sub>2</sub> give rise to 12 volumes of CO<sub>2</sub>. Of these 12 volumes only 9 are formed by a direct union with atmospheric O<sub>2</sub>, and only these 9 could cause reduction in volume or pressure in a closed space; the other 3 volumes of CO<sub>2</sub> came from the breakdown of the citric acid molecule, and could cause no reduction in pressure, even if they subsequently went into solution or were combined in some way.

Applying this information to the present problem, it appears that the CO<sub>2</sub> produced in excess of the O<sub>2</sub> used up can not cause reduction in pressure, and that consequently we should deduct from the values for the gain in CO<sub>2</sub> in the tissue the volumes of CO<sub>2</sub> formed in excess of the volumes of O<sub>2</sub> used up in that particular test. The difference represents CO<sub>2</sub> that was formed as a result of a union with atmospheric O<sub>2</sub>, and is the fraction that was available for causing reduced pressure upon dissolving or combining in the tissue. This is the basis for the entries in column 7, Table II, and column 5, Table III. As an example of the computation let us take the entry in line 1, Table II:

$$\text{Total CO}_2 \text{ produced} = 367 + 86 = 453 \text{ cc.}$$

$$\text{Total O}_2 \text{ consumed} = 421 + (-.6) = \underline{420 \text{ cc.}}$$

$$\text{Excess CO}_2 \text{ not formed by combining with atmospheric O}_2 = 33 \text{ cc.}$$

$$\text{CO}_2 \text{ gain in tissue} = 86 \text{ cc.}$$

$$\text{Excess CO}_2 = 33 \text{ cc.}$$

$$\text{CO}_2 \text{ resulting from combination with atmospheric O}_2 = 53 \text{ cc.}$$

Thus, in this case only 53 cc. of the gain in tissue CO<sub>2</sub> were formed by combination with atmospheric O<sub>2</sub>, and only this amount could be available for reducing the pressure if it became dissolved or combined. The reduced pressure actually observed for this lot was 43 cc., and so the per cent accounted for was  $100 (43 \div 53) = 81$ . It is in this way that the other values in column 7, Table II, and column 5, Table III, were computed (except, of course, for such entries as showed no excess of CO<sub>2</sub>, i.e., with R. Q. values of 1.00 or less, no corrections being applied in such cases).

It is not claimed that in the present experiments the substrates furnishing the excess CO<sub>2</sub> (R. Q. values higher than 1.00) were malic acid, or citric acid, or that the oxidation was complete in any case. But whatever the substrate may have been, if the R. Q. was greater than 1.00, the extra CO<sub>2</sub> was not derived from a combination with atmospheric O<sub>2</sub>, and consequently could not have been available for bringing about a reduced pressure in the containers.

A consideration of the problem from this point of view led to the ratios in column 10, Table II, and column 8, Table III, which show the percentage of the CO<sub>2</sub> gain in the tissue accounted for by the reduced pressure after correction for the excess CO<sub>2</sub> formed over the amount of O<sub>2</sub> consumed.

An estimate of the experimental error can be made from the replicates (lots run simultaneously) of which there were available the following: for sweet-potato, one set of three entries, and two sets of four entries each; for apple and potato, four entries each. These furnished 14 degrees of freedom

for experimental error. The standard deviation was found to be 16.0, and the coefficient of variation was  $16 \div 91.2 = 17.5$  per cent. The range for the mean of each tissue was then found by using as a factor the "t" value corresponding to a probability of 0.05 with 14 DF. The values obtained were: for tomato 87 to 115; for turnip 75 to 109; for sweet-potato 78 to 98; for apple 69 to 97; for potato 71 to 106. This range may be high for the tomato data, the average being influenced strongly by the value 155 in line 5. If this item is omitted in the computation, the tomato range becomes 77 to 105, values about the same as those obtained for the other tissues. Thus, it appears that the values may be as low as 70 to 80. The distribution of these values is skew on the basis of the test described by Snedecor (6, p. 174). The median is 84, and the mode at about 80.

Taking into consideration the values for the median, the mode, and the range of the means, it is concluded that about 75 to 85 per cent of the  $\text{CO}_2$  gain in the tissue, after correction for the excess  $\text{CO}_2$  produced over  $\text{O}_2$  consumed, has been accounted for by the reduced pressure in the container.

This leaves about 15 to 25 per cent unaccounted for. It is believed that this represents  $\text{CO}_2$  remaining as a gas in the tissue without having become dissolved or combined in any way. This would be estimated as  $\text{CO}_2$  by the aeration method of estimating  $\text{CO}_2$  in the tissue, but, of course, not having become dissolved or combined it could not function in reducing the pressure in the container.

#### VOLUME PERCENTAGES OF GAS FRACTIONS AT THE END OF TESTS

An effort was made to adjust the amount of tissue used in each test, and the duration of the experimental period, so that a good supply of  $\text{O}_2$  would still be available in the air in the desiccator at the end. The object was to avoid the occurrence of any anaerobic respiration. The amounts of  $\text{O}_2$  remaining in the desiccators at the end of each test are shown (to the nearest tenth per cent) in column 3, Table IV. The volume percentage of  $\text{O}_2$  at the end was greater than 10 per cent in 26 out of the 32 experiments and was below 6 per cent in only two tests; but even with the lowest amount of  $\text{O}_2$ , 4.5 per cent, probably no anaerobic respiration occurred. The volume percentages of  $\text{CO}_2$  at the end of each test are shown in column 4, Table IV, expressed to the nearest tenth per cent.

The balance of Table IV is devoted to furnishing evidence on a subsidiary problem, only indirectly connected with the principal objects of these tests, but one of considerable interest regarding the different gas fractions involved in a respiration experiment with tissue in a closed space. The problem relates to the sum of the volume percentages of the various gas fractions, and the relation this bears to the original percentage of  $\text{O}_2$  in the air at the start, i.e. 20.9 per cent.

TABLE IV  
VOLUME PERCENTAGE RELATIONSHIPS OF GAS FRACTIONS IN AIR AND IN  
TISSUE AT THE END OF EACH TEST

Tissue	Corresponding lot in Tables II & III	% by vol. in container at end		CO <sub>2</sub> gain in tissue as % of container volume	Excess CO <sub>2</sub> as % of container volume	Sum of Cols. 3, 4, 5 minus Col. 6
Tomato	Table II line 1	10.6	9.2	2.2	0.8	21.2
	" " " 2	7.2	13.1	3.1	2.3	21.1
	" " " 3	11.2	8.2	1.9	0.0	21.3
	" " " 4	8.7	10.5	2.8	0.8	21.2
	" " " 5	12.0	8.6	1.6	1.1	21.1
	" " " 6	9.9	9.9	2.2	0.9	21.1
Turnip	Table II line 7	11.8	6.8	3.3	0.3	21.6
	" " " 8	5.6	11.8	4.6	1.1	20.9
	" " " 9	6.7	12.0	3.6	0.2	22.1
	" " " 10	4.5	12.7	5.7	0.0	22.9
Apple	Table II line 11	18.0	2.4	0.7	0.0	21.1
	" " " 12	14.3	5.6	1.6	0.0	21.5
	Table III " 12	15.2	5.0	2.1	1.3	21.0
	" " " 13	15.3	4.9	2.1	1.2	21.1
	" " " 14	15.8	4.3	1.9	1.0	21.0
	" " " 15	16.3	4.9	2.3	1.0	22.5
Sweet- potato	Table II line 13	14.7	4.7	1.7	0.0	21.1
	Table III line 1	13.5	6.9	1.9	1.0	21.3
	" " " 2	13.7	6.9	1.9	0.8	21.7
	" " " 3	13.0	5.7	1.7	0.0	20.4
	" " " 4	11.7	8.0	2.4	1.0	21.1
	" " " 5	11.9	7.7	2.6	1.1	21.1
	" " " 6	10.2	9.4	3.1	1.6	21.1
	" " " 7	10.6	9.0	3.1	1.6	21.1
	" " " 8	14.5	5.4	1.4	0.0	21.3
	" " " 9	11.3	8.4	2.6	1.0	21.3
	" " " 10	10.2	9.2	2.5	0.7	21.2
	" " " 11	10.9	9.0	2.6	1.5	21.0
Potato	Table III line 16	16.3	3.5	1.1	0.0	20.9
	" " " 17	16.3	3.4	1.3	0.0	21.0
	" " " 18	16.2	3.6	1.4	0.1	21.1
	" " " 19	16.4	3.3	1.0	0.0	20.7

If the R. Q. is 1.00 and if none of the CO<sub>2</sub> produced were absorbed by the tissue, the sum of the volume percentages of CO<sub>2</sub> and O<sub>2</sub> would be 20.9. If, however, some of the CO<sub>2</sub> produced was absorbed by the tissue the sum of the percentages of CO<sub>2</sub> and O<sub>2</sub> would be less than 20.9; but if the R. Q. was more than 1.00, and if the excess CO<sub>2</sub>, or some of it, was not absorbed by the tissue the sum of the percentages would tend to be increased to more than 20.9 by this factor. From the results of the present experiments we can get estimates of these last two forces which cause deviation from the sum 20.9. The gains in CO<sub>2</sub> in the tissue are shown in column 6, Table II and column 4, Table III, and by referring these amounts to the net volume of the space in the container, the per cent by volume of the gain in tissue

CO<sub>2</sub>, if it were present as a gas in a space of that size, can be computed. These values are shown in column 5, Table IV. Likewise, the excess CO<sub>2</sub> produced over the amount of O<sub>2</sub> consumed can be found by subtracting column 7 from column 6 in Table II, and column 5 from column 4 in Table III. These differences when related to the net volume in the container show the percentage by volume of the excess of CO<sub>2</sub> acting to increase the total percentage above 20.9. These values are shown in column 6, Table IV.

For our purpose we wish to know the sum of the values in columns 3, 4, and 5 minus the values in column 6, and to compare this value with 20.9. The results are shown in the right hand column in Table IV. The average is 21.25. The distribution is somewhat skew, however. The mode is 21.1 and the median is about 21.14. Of the 32 entries 18 are either 21.0, 21.1 or 21.2. The uniformity of the values was unexpected; the coefficient of variation is less than one per cent.

Although the value 20.9 was definitely exceeded, indicating a systematic error either in over-estimating CO<sub>2</sub>, or in under-estimating O<sub>2</sub>, the divergence is not large. Thus, the excess over 20.9 of the average is 0.35 per cent, and of the median is 0.24 per cent. On a net volume of air space amounting to, say, 4000 cc., the divergence would be 10 to 14 cc.

#### CHANGES IN O<sub>2</sub> CONTENT OF TISSUES AND THE SURROUNDING AIR

In previous experiments (1) with potato tubers respiring in closed containers, it was found that while it was very important to determine the change in the CO<sub>2</sub> content of the tissue during the experimental interval, this was not the case with the O<sub>2</sub> content of the tissue. The change in the amount of O<sub>2</sub> in the tissue was so small in proportion to the change in O<sub>2</sub> which occurred in the surrounding air that it could be neglected without having any important effect upon the measurement of the total amount of O<sub>2</sub> used up during the interval.

The present tests furnish evidence on this point for tomato, turnip, apple, and sweet-potato (see columns 3 and 4, Table II). The total amount of O<sub>2</sub> used up in each test is the sum of the entries in columns 3 and 4. The change in the O<sub>2</sub> content of the surrounding air constituted nearly all of this total. If the values in column 3 are expressed as a percentage of the sum of columns 3 and 4, it will be found that the average percentage is 99.3. That is, the O<sub>2</sub> loss as measured by the analysis of the surrounding air would have given the true value within an error of less than one per cent. In only one of the 13 tests was this error greater than one per cent; and that was with the apple lot in line 11, columns 3 and 4, Table II, in which case it was 4.6 per cent.

#### SUMMARY

Roots of turnip and sweet-potato, fruits of apple and tomato, and tubers of potato were placed in containers that were then sealed. Respiration

was permitted to continue at 20° C. for duration periods such that ample supplies of oxygen would still remain at the end of the test. Measurements were made not only of the change in O<sub>2</sub> and CO<sub>2</sub> in the air surrounding the tissue and in the tissues themselves, but also of the reduction in pressure produced in the container by the action of the tissues during the period of respiration.

Presumably the reduction in pressure in the container is caused by the absorption by the tissue of carbon dioxide formed during respiration, but which is subsequently either dissolved in the plant sap, or is combined in some way with tissue constituents. It was found that the amount of reduced pressure accounted for 80 to 87 per cent of the gain in CO<sub>2</sub> in the tissue in the case of turnip and potato, but for less than 60 per cent in the case of tomato and sweet-potato, and for less than 50 per cent with apple.

This difference in the response of the different tissues was related to the respiration quotient (volume of CO<sub>2</sub> produced ÷ volume of O<sub>2</sub> consumed). The R. Q. was about 1.00 for potato and turnip, but was in the range 1.08 to 1.12 for the other tissues. Arguments are offered to the effect that only the CO<sub>2</sub> which results from a combination with atmospheric O<sub>2</sub> in these circumstances can bring about a reduction in pressure in the container when this CO<sub>2</sub> is absorbed by or re-combined with the tissue. The extra CO<sub>2</sub> produced over O<sub>2</sub> consumed in the tests in which the R. Q. was greater than 1.00, was derived directly from the molecules of the substrates undergoing oxidation, was not formed by union with atmospheric O<sub>2</sub>, and could not cause reduction in volume even if it had later been dissolved or re-combined (although it could increase the volume or pressure if it was not dissolved or combined).

When the data were reconsidered from this point of view, and when the amount of the extra CO<sub>2</sub> produced in excess of the O<sub>2</sub> consumed was deducted from the CO<sub>2</sub> gain in the tissue, the differences so obtained were then compared with the amount of the reduced pressure found in each of the tests. On this basis the response was fairly similar for the different tissues. About 75 to 85 per cent of the gain in CO<sub>2</sub> in the tissue was accounted for by the reduced pressure observed.

The balance unaccounted for, i.e., about 15 to 25 per cent of the CO<sub>2</sub> gain in the tissue, is believed to be CO<sub>2</sub> remaining in the tissue as a gas without having been dissolved or combined with the tissue in any way.

The change in the oxygen content of the tissues undergoing respiration was small in comparison to the change in the oxygen content of the air surrounding the tissue. Of the total amount of oxygen consumed in a given test about 99 per cent, or more, was derived from the oxygen of the air surrounding the tissue. In experiments which have for their object the determination of the oxygen consumed over a given period, it would be ordinarily unnecessary even with the tissue in a closed space, to take into account any change in the oxygen content of the tissue between the be-

ginning and the end of the test. This is in contrast to the situation with carbon dioxide which under similar conditions tends to accumulate in the tissue, and which must be estimated in both the tissue and in the surrounding air, in order that correct values for the total amount of carbon dioxide produced may be obtained.

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# SOME CYCLIC ACETALS CONTAINING THE 3,4-METHYLENE-DIOXYPHENYL RADICAL AND THEIR INSECTICIDAL EFFECTIVENESS AGAINST HOUSEFLIES

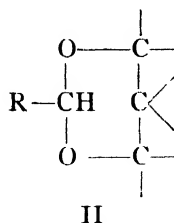
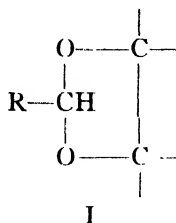
EDWARD A. PRILL, ALBERT HARTZELL, AND JOHN M. ARTHUR

In the past few years, certain compounds of various types containing the 3,4-methylenedioxyphenyl radical  $[3,4-(\text{CH}_2\text{O}_2)\text{C}_6\text{H}_3\cdot]$  in their structures have been found to be insecticidally active. As a rule, the active compounds containing this radical are most effective when used together with a small amount of pyrethrum, with which they exhibit synergistic action.

The present paper deals with some compounds containing this radical linked to a cyclic acetal ring (or in a few cases, an analog of a cyclic acetal ring). These compounds and their data are given in Table I. Several were found to be quite effective against houseflies.

## PREPARATION OF COMPOUNDS

*Cyclic acetals of piperonal and analogs of these.* As a general rule, under conditions favorable for the removal of water, aldehydes ( $\text{R}-\text{CHO}$ ) react with glycols, which have their hydroxyl groups separated by two or three carbon atoms, to form cyclic acetals, designated, respectively, as 1,3-dioxolane derivatives (I), and 1,3-dioxane derivatives (II).



When piperonal  $[3,4-(\text{CH}_2\text{O}_2)\text{C}_6\text{H}_3-\text{CHO}]$  is used as the aldehyde, such derivatives, in which the R in position 2 is a methylenedioxyphenyl radical, should be formed.

Cyclic acetals of piperonal were easily prepared by adaptation of the general method used by Senkus (10) for cyclic acetals of other fairly high boiling aldehydes. A mixture of 0.1 mole of piperonal, somewhat more than 0.1 mole of a glycol, 0.5 g. of *p*-toluenesulphonic acid as a catalyst, and about 75 ml. of benzene was heated in a flask fitted with a Dean and Stark (1) type moisture trap attached to a reflux condenser so that the water produced in the reaction was continuously removed. (In making Compound 2 the pinacol hydrate used as the glycol was dehydrated in the apparatus



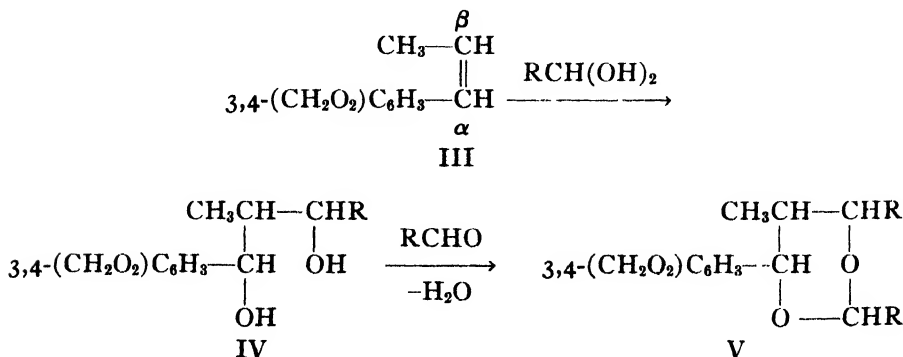
before adding the acid catalyst and piperonal.) After completion of the reaction the cooled benzene solution was washed with dilute aqueous alkali and then with water. After removal of the solvent the product was fractionally distilled under reduced pressure unless it was too high boiling or it could be crystallized. Sulphur and nitrogen analogs of cyclic acetals (Compounds 9 and 12) were similarly prepared by using a dithiol or an aminoalcohol in place of a glycol. The formulas of the intermediates used in the reactions with piperonal to produce Compounds 1 through 13 of Table I are readily made apparent by replacing the 3,4-methylenedioxybenzylidine radical in the formula of each of these products by two hydrogen atoms.

Compound 14 was prepared from Compound 13 by catalytic reduction of the nitro group followed by acylation with *n*-caproyl chloride.

Compound 15 was prepared from 6-bromopiperonal (5) and 2-methyl-2,4-pentanediol by the general method.

Compounds 1, 2, 3, 6, and 15 appear to be among the most stable on storage. Some of the other compounds are less stable since the odor of free piperonal became distinct on storage. Compound 4, made with 2-ethyl-1,3-hexanediol (this glycol is the insect repellent "Rutgers 612") appears to be particularly unstable; one batch of it decomposed on distillation at 2 mm.; another batch was successfully distilled but it turned dark and odorous after a few weeks.

*Products of isosafrole and aliphatic aldehydes.* Compounds of a type somewhat related to the preceding compounds were made from isosafrole and simple aliphatic aldehydes through the use of a reaction discovered by Prins (8, 9) in his study of the behavior of certain vinyl- and propenyl-aryl compounds with formaldehyde. In this reaction it may be visualized that the  $\text{-OH}$  and the  $\text{-CHROH}$  radicals of the hydrate of the aldehyde,  $\text{RCH(OH)}_2$ , add across the  $\alpha, \beta$  double bond of isosafrole (III) to form a glycol (IV), which reacts with a second molecule of the aldehyde to form a cyclic acetal designatable as a 4-(3,4-methylenedioxyphenyl)-1,3-dioxane derivative (V).



Thus with formaldehyde (R is H) the 5-methyl derivative (Compound 16) was obtained, and with acetaldehyde (R is CH<sub>3</sub>) the 2,5,6-trimethyl derivative (Compound 17). The order of the primary addition of the radicals of an aldehyde hydrate to the  $\alpha$  and  $\beta$  carbon atoms of isosafrole is assumed to be the same as that shown to be true in more recent studies (2, 3) of similar cases involving the addition of such radicals to the  $\alpha$  and  $\beta$  carbon atoms of styrene, and to be the reverse from the order originally postulated by Prins.

Compound 16, previously prepared by Prins (8, 9), was made by a modification of his method in that an excess of formaldehyde was used. A stoppered pressure bottle containing a mixture of 40 g. of isosafrole, 100 ml. of 37 per cent formaldehyde, and 120 g. of concentrated sulphuric acid diluted with 200 ml. of water, was shaken on a shaking machine for about 24 hours. The product was extracted with ethyl ether, washed with dilute aqueous alkali, and dried over anhydrous sodium sulphate. After removal of the solvent, the compound was separated by fractional distillation at 2 mm. and obtained in fairly good yield as a very viscous oil. One vial of it happened to become converted into crystals and other portions could be induced to crystallize by seeding.

Compound 17 was prepared in a similar manner by shaking a mixture of 20 g. of isosafrole, 45 g. of paraldehyde (source of acetaldehyde) and 100 ml. of 30 per cent (by wt.) sulphuric acid. The conversion in this case was much slower. The purified compound (yield 5 g.) is an extremely viscous oil. It is completely soluble in "Deo-base," while Compound 16 requires a little co-solvent for solution.

Propionaldehyde and *n*-butyraldehyde could not be made to react with isosafrole by prolonged shaking with 30 per cent sulphuric acid.

#### METHOD OF TESTING

The compounds were tested against houseflies (*Musca domestica* L.) by the large group Peet-Grady method as recommended by the National Association of Insecticide and Disinfectant Manufacturers (6). Control tests with the regular Official Test Insecticide (O.T.I.), which is supplied by the above Association and which contains about 0.1 g. pyrethrins per 100 ml., were performed on each culture of flies used. Test solutions were prepared by dissolving the compounds in "Deo-base" (a purified petroleum distillate) if possible, otherwise in a mixture of "Deo-base" and acetone. Usually pyrethrum extract equivalent to 0.025 g. pyrethrins per 100 ml. was included in the test solutions to provide adequate knockdown and to make possible manifestations of any synergistic activity which the compounds may possess. The results are given in Table I.

#### DISCUSSION OF RESULTS

Compounds 1, 2, 5, and 6, which were made with 2-methyl-2,4-pentanediol, pinacol, glycerol  $\alpha$ -*n*-butyl ether, and 2,3-butanediol, respectively

TABLE I

SOME CYCLIC ACETALS CONTAINING THE 3,4-METHYLENEDIOXYPHENYL RADICAL AND THE RESULTS OF PEET-GRADY TESTS ON THESE, USUALLY WITH ADDED PYRETHRINS

CONCENTRATION OF PYRETHRINS INCORPORATED INTO TEST SOLUTIONS:  
0.025 G. PER 100 ML., UNLESS OTHERWISE INDICATED

No.	Compound Probable formula where M is 3,4-(CH <sub>2</sub> O) <sub>2</sub> C <sub>6</sub> H <sub>3</sub> —	B.p. ° C./mm.	Concn., g. per 100 ml.	24-Hr. kill, %	O.T.I. kill, %
1	$  \begin{array}{c}  \text{O} - \text{CHCH}_3 \\    \quad   \\  \text{M} - \text{CH} \quad \text{CH}_2 \\    \quad   \\  \text{O} - \text{C}(\text{CH}_3)_2  \end{array}  $	133-135/2	2.0 0.75* 0.75 0.5 0.25	99 43 (K.d. 90) 73 55 46	48 43 43 46 46
2	$  \begin{array}{c}  \text{O} - \text{C}(\text{CH}_3)_2 \\    \quad   \\  \text{M} - \text{CH} \quad   \\    \quad   \\  \text{O} - \text{C}(\text{CH}_3)_2  \end{array}  $	128-130/1	0.75* 0.75 0.5 0.25	54 (K.d. 95) 87 76 64	50 50 44 45
3	$  \begin{array}{c}  \text{O} - \text{C}(\text{CH}_3)(\text{C}_2\text{H}_5) \\    \quad   \\  \text{M} - \text{CH} \quad   \\    \quad   \\  \text{O} - \text{C}(\text{CH}_3)(\text{C}_2\text{H}_5)  \end{array}  $	150-152/1	0.5 0.25	76 52	61 61
4	$  \begin{array}{c}  \text{O} - \text{CH}_2 \\    \quad   \\  \text{M} - \text{CH} \quad \text{CHCH}_2\text{CH}_3 \\    \quad   \\  \text{O} - \text{CHCH}_2\text{CH}_2\text{CH}_3  \end{array}  $	165-167/2	1.0 0.5	61 42	45 45
5	$  \begin{array}{c}  \text{O} - \text{CH}_2 \\    \quad   \\  \text{M} - \text{CH} \quad   \\    \quad   \\  \text{O} - \text{CHCH}_2 - \text{O} - (\text{CH}_2)_3\text{CH}_3  \end{array}  $	165-168/2	0.75* 0.75 0.5 0.25	54 (K.d. 90) 81 82 63	45 41 43 45
6	$  \begin{array}{c}  \text{O} - \text{CHCH}_3 \\    \quad   \\  \text{M} - \text{CH} \quad   \\    \quad   \\  \text{O} - \text{CHCH}_3  \end{array}  $	120-123/1	1.0 0.5 0.25	90 65 41	45 50 45
7	$  \begin{array}{c}  \text{O} - \text{CH}_2 \\    \quad   \\  \text{M} - \text{CH} \quad   \\    \quad   \\  \text{O} - \text{CH}_2  \end{array}  $	112-116/2	2.0	45	45
8	$  \begin{array}{c}  \text{O} - \text{CH}_2 \\    \quad   \\  \text{M} - \text{CH} \quad \text{CH}_2 \\    \quad   \\  \text{O} - \text{CH}_2  \end{array}  $	139-141/2	1.0	37	41
9	$  \begin{array}{c}  \text{S} - \text{CH}_2 \\    \quad   \\  \text{M} - \text{CH} \quad \text{CH}_2 \\    \quad   \\  \text{S} - \text{CH}_2  \end{array}  $	(M.p. 88-89°)	0.5	77	61

TABLE I (Continued)

No.	Compound Probable formula where M is 3,4-(CH <sub>2</sub> O) <sub>2</sub> C <sub>6</sub> H <sub>3</sub> —	B.p. ° C./mm.	Concn., g. per 100 ml.	24-Hr. kill, %	O.T.I. kill, %
10	$\begin{array}{c} \text{O} \cdots \text{CH}_2 \\   \\ \text{M}-\text{CH} \\   \\ \text{O}-\text{CHCH}_2\text{Cl} \end{array}$	150-153/1	0.5	65	61
11	$\begin{array}{c} \text{O}-\text{CH}-\text{CO}_2\text{C}_2\text{H}_5 \\   \\ \text{M}-\text{CH} \\   \\ \text{O}-\text{CH}-\text{CO}_2\text{C}_2\text{H}_5 \end{array}$	Oil (crude)	1.0	6	30
12	$\begin{array}{c} \text{O}-\text{CH}_2 \\   \\ \text{M}-\text{CH} \\   \\ \text{CH}_3(\text{CH}_2)_3-\text{N}-\text{CH}_2 \end{array}$	150 154/3	1.0	37	49
13	$\begin{array}{c} \text{O}-\text{CH}_2 \\   \\ \text{M}-\text{CH} \quad \text{C}(\text{CH}_3)(\text{NO}_2) \\   \\ \text{O}-\text{CH}_2 \end{array}$	(M.p. 150°)	0.5	47	56
14	$\begin{array}{c} \text{O}-\text{CH}_2 \\   \\ \text{M}-\text{CH} \quad \text{C}(\text{CH}_3) \cdot \text{NHCOC}_6\text{H}_{11} \\   \\ \text{O}-\text{CH}_2 \end{array}$	Gum (crude)	0.5* 0.5 0.2 0.1	66 (K.d. 96) 87 71 54	44  43 48 48
15	$\begin{array}{c} \text{O}-\text{CHCH}_3 \\   \quad   \\ 3,4-(\text{CH}_2\text{O})_2-6-\text{Br}-\text{C}_6\text{H}_2-\text{CH} \quad \text{CH}_2 \\   \quad   \\ \text{O}-\text{C}(\text{CH}_3)_2 \end{array}$	162-164/2	2.0 0.75	65 52	46 41
16	$\begin{array}{c} \text{CH}_3\text{CH}-\text{CH}_2 \\   \quad   \\ \text{M}-\text{CH} \quad \text{O} \\   \quad   \\ \text{O}-\text{CH}_2 \end{array}$	141 143/2 (M.p. 37°)	1.0* 1.0 0.5* 0.5	72 (K.d. 99) 99 24 (K.d. 88) 63	56  56 54 55
17	$\begin{array}{c} \text{CH}_3\text{CH}-\text{CHCH}_3^{**} \\   \quad   \\ \text{M}-\text{CH} \quad \text{O} \\   \quad   \\ \text{O}-\text{CHCH}_3 \end{array}$	145-147/2	1.0* 1.0 0.5 0.25	92 (K.d. 98) 94 79 50	53  44 47 45

\* In these cases no pyrethrins were incorporated into the test solutions; the percentages of the flies knocked down in 10 minutes are indicated in parentheses (K.d.). In all other cases the 10-minute knockdown values are not indicated since they were at least over 90 per cent by virtue of the pyrethrins present.

\*\* Calcd. for C<sub>14</sub>H<sub>18</sub>O<sub>4</sub>: C, 67.2%; H, 7.22%. Found C, 66.1%; H, 7.35%. This compound was analyzed by Miss Elisabeth Heber-Smith.

were found to be the most effective of the cyclic acetals made directly with simple glycols and piperonal. Compound 14, a more complex cyclic acetal which also contains an amide structure, was found to be particularly effective. Compounds 16 and 17, in which the methylenedioxyphenyl radical is in a different position on the cyclic acetal ring as compared structurally with the cyclic acetals of piperonal, were also found to be quite effective.

Synergistic action of Compounds 1, 2, 5, 14, and 16 with pyrethrins was demonstrated in each case by testing a certain concentration of the compound without pyrethrins as well as with 0.025 g. of pyrethrins per 100 ml. Since this concentration of pyrethrins alone commonly gave a 24 hour kill of only about 20 per cent, it is apparent that the kills obtained with the mixtures were more than additive. (Synergistic action also probably could be demonstrated with Compound 17 if tested at a lower concentration without pyrethrins.)

In Compound 15, which is a brominated derivative of Compound 1, the bromine substituent causes reduction of activity.

Some of the compounds tested showed little or no activity. The mere presence of a methylenedioxyphenyl radical in the structure is not sufficient for activity (4, 7).

It appears significant that whereas piperonal itself is useless against houseflies (4, 7), although it has been recommended for the destruction of lice (11, p. 1525), certain cyclic acetals of piperonal are quite effective against houseflies. On the basis of the compounds studied, it appears that of the simple glycols, those containing about six carbon atoms, on reaction with piperonal, often produce the more active cyclic acetals. It was also noted that the aliphatic glycols containing at least six carbon atoms and no extra oxygen form cyclic acetals with piperonal which are completely soluble in "Deo-base."

Cyclic acetals of benzaldehyde, *o*- and *p*-chlorobenzaldehydes, anisaldehyde, 3,4-diethoxybenzaldehyde, cinnamaldehyde, and furfural with 2-methyl-2,4-pentanediol which were prepared similarly to the cyclic acetals of piperonal, were found on testing (results not included in the table) to be practically inactive against houseflies at a concentration of 2 g. per 100 ml. with the usual small amount of pyrethrins added. These compounds do not contain a methylenedioxyphenyl radical.

#### SUMMARY

A number of compounds containing the 3,4-methylenedioxyphenyl radical linked to a cyclic acetal ring have been prepared and tested, usually in admixture with a low concentration of pyrethrins, for insecticidal effectiveness against houseflies (*Musca domestica* L.) by the Peet-Grady method.

Of fourteen cyclic acetals of piperonal studied (including two analogs

in which oxygen of the cyclic acetal ring is replaced by sulphur or by nitrogen) the most active were found to be those formed from 2-methyl-2,4-pentanediol, pinacol, glycerol  $\alpha$ -*n*-butyl ether, and 2,3-butanediol, by reaction with piperonal, and also a more complex cyclic acetal, 2-(3,4-methylenedioxyphenyl)-5-methyl-5-*n*-caproylamino-1,3-dioxane.

The cyclic acetal of 6-bromopiperonal and 2-methyl-2,4-pentanediol was found less active than the cyclic acetal of piperonal and this glycol.

The 5-methyl and the 2,5,6-trimethyl derivatives of 4-(3,4-methylenedioxyphenyl)-1,3-dioxane, made by the reactions of isosafrole with formaldehyde and with acetaldehyde, respectively, were also found quite active.

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## GERMINATION AND SEX POPULATION STUDIES OF ILEX OPACA AIT.

LELA V. BARTON AND NORWOOD C. THORNTON<sup>1</sup>

### INTRODUCTION

These experiments were designed to test the potentialities and behavior of the seeds of different sizes contained in the American holly fruit (*Ilex opaca* Ait.). It has been reported that each berry contains two "large" seeds and two "small" seeds and that the "large" seeds always produce pistillate or female plants while the "small" seeds always produce staminate or male plants.

To test the validity of this claim as well as to secure germination data, fruits were harvested from the Institute farm in Yonkers, New York, in November, 1933, and December, 1934. The fruits were apparently ripe when collected, all of them having a deep red color. The berries were macerated individually and the two large seeds separated from the two small seeds. It was necessary to take the fruits one by one for the large seeds of one fruit were no larger than the small seeds of another fruit. Similarly the small seeds of some of the berries were comparable in size to the large ones of other berries. Because the two small seeds in some berries were intermediate in size between the largest and smallest seeds, they were classified as medium. Thus some fruits had two large and two medium seeds while others had two large and two small seeds. Many of the individual fruits contained four seeds of approximately equal size and many more had three seeds of one size and one smaller seed. Where there was doubt about the separation into two sizes the seeds were discarded. Occasionally fruits were found with only three seeds and more often there were those with five or six seeds. The seeds from the latter were kept as a separate lot as reported below. A total of 12,000 seeds was used in the tests.

The seeds were planted in soil as soon as they were cleaned and graded for size. It was recognized that no advantage would accrue to fall planting over that to be gained by spring planting (1, 2, 3). However, fall planting has not been considered harmful to the germination of the seeds (1, 2) and the mechanics of the experiment were facilitated by this procedure. A soil mixture of equal parts of sod soil, granulated peat moss, and sand was used. For the most part three hundred seeds were planted in each flat though some flats contained only half that number.

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## RESULTS AND DISCUSSION

## GERMINATION

Summarized results of the germination tests are to be found in Tables I and II. For convenience in presentation all of the data for one planting date for each seed size have been combined. No seedlings appeared in any of the flats the first spring after planting. The second spring seedlings appeared above ground. There was no uniformity however in the actual number of seedlings produced. For example the different plantings of the large seeds yielded from 7 to 36 per cent seedlings after two winters in the soil.

TABLE I

GERMINATION OF HOLLY SEEDS PLANTED IN SOIL IN FLATS AND KEPT IN A COLD FRAME. MULCHED OVER WINTER

Size of seed	Seed planting date	No. of seeds planted	Per cent seedling production	
			After 2 winters	After 3 winters
Large	Nov. 10, 1933	1200	7	41
	Dec. 8, 1934	1200	36	53
	Dec. 13, 1934	1500	29	65
	Dec. 20, 1934	1800	12	65
	Total	5700	21	57
Medium	Dec. 8, 1934	600	15	64
	Dec. 13, 1934	600	35	61
	Dec. 20, 1934	600	25	66
	Total	1800	25	64
Small	Nov. 10, 1933	1200	5	26
	Dec. 8, 1934	600	1	18
	Dec. 13, 1934	900	3	21
	Dec. 20, 1934	900	3	27
	Total	3600	3	24
Mixed (5 or 6 seeds per berry)	Dec. 13, 1934	600	1	32
	Dec. 20, 1934	300	1	63
	Total	900	1	42

More seedlings appeared after a third winter. There was much less variation in the germination percentages obtained from different flats of any given lot at this time. When the totals for the different sizes of seeds are compared (Table I), it is seen that very much higher seedling production was secured from medium and large seeds (64 and 57 per cent) than from the small seeds (24 per cent). This may have been due to the fact that many of the small seeds were empty or contained embryos of very low vitality.

There was some indication that the 1933 crop of large seeds may have been inferior in quality to the 1934 collections, since only 41 per cent of the former produced seedlings after three winters while 53 to 65 per cent of the

latter germinated. The corresponding small seeds, however, were equal to those harvested in 1934 in germinating ability.

The two plantings of mixed lots taken from berries containing five or six seeds each differed greatly in their germination behavior, one lot exhibiting twice the germinating capacity of the other.

When the data were rearranged to show the effect of seed lot or planting date on subsequent seedling production the 1933 crop again made a poor showing (Table II). Whether this was due to the earlier harvest, to poor quality seeds, or to the environmental conditions which prevailed after planting, is a matter of conjecture. It has already been seen from Table I that this poor performance can be attributed to the large seeds rather than the small seeds in the berries. This might indicate a lack of full

TABLE II  
GERMINATION OF HOLLY SEEDS PLANTED IN SOIL IN FLATS AND KEPT IN A  
COLD FRAME. MULCHED OVER WINTER

Seed planting date	No. of seeds planted	Per cent seedling production	
		After 2 winters	After 3 winters
Nov. 10, 1933	2400	6	33
Dec. 8, 1934	2400	22	47
Dec. 13, 1934	3600	19	48
Dec. 20, 1934	3600	11	56

development of the large seeds of this particular crop at harvest, which was at least one month earlier than for any of the 1934 collections. It should be stated here that the planting date indicates the approximate harvest date since all seeds were planted within four days after collection.

The grand total of seeds used was 12,000. Seedlings numbering 1757 were produced after two winters and 5654 appeared after three winters. The final germination percentage was 47 when all classes and plantings were considered together. This is approximately the percentage that has been reported by others. The data in Table I indicate that at least part of the low germination percentage can be traced to the small, and therefore probably poorly developed, seeds in the fruit. In nature, however, a very much smaller germination percentage has been reported. Ives (2, p. 73) stated: "The ratio of germination in nature, under favorable conditions, is not over 1:1,000,000, while the average for the range probably would be about 1:10,000,000." He listed a tough woody pericarp, an immature embryo and low water supply as causes of germination failures.

#### POPULATIONS OF STAMINATE AND PISTILLATE PLANTS

In May 1939, 1255 of the seedlings produced from the germination

tests described above were set in the field. These were selected from lots representing the different seed sizes and planting dates. Also both two-year-old and three-year-old seedlings were transplanted. The primary purpose here was to find whether any relation existed between the size of the seed and the sex of the seedling produced.

The first plants flowered in 1942. Of a total of 25 which bloomed at that time 23 were staminate and 2 pistillate. The following year new plants flowered bringing the total to 48 of which 41 were staminate and 7 pistillate. The great preponderance of male plants among the early flowering individuals was not correlated in any way with seed size.

TABLE III  
HOLLY FLOWERING DATA AS OF 1947

Criterion for comparison		Total No. of seedlings in field	Per cent of seedlings flowering	Per cent of flowering seedlings	
				Staminate	Pistillate
Seed size	Large	543	46	64	36
	Medium	383	23	62	38
	Small	291	23	68	32
	Mixed*	38	0	0	0
Age of seedlings when set in field	2 years	683	24	66	34
	3 years	572	42	63	37
Planting date of seeds	Nov. 10, 1933	189	23	67	33
	Dec. 8, 1934	300	36	66	34
	Dec. 13, 1934	367	30	61	39
	Dec. 20, 1934	399	36	63	37
Total		1255	32	64	36

\* 5 or 6 seeds from each berry.

By 1944, 193 plants had flowered: 130 staminate and 63 pistillate. This meant a ratio of 67 per cent male to 33 per cent female plants. Essentially this same ratio has been maintained since that time. Of the total of 224 plants which had bloomed by 1946, 69 per cent were staminate and 31 per cent pistillate.

The flowering data as of 1947 are to be found in Table III. The data have been arranged to permit comparisons using seed size, age of seedlings when set in the field, and planting date of the seeds as criteria.

In a consideration of the effect of seed size upon sex of the seedlings, the medium and small seeds should be combined as a unit to compare with the large seeds. However, this is not necessary since seedlings from all seeds regardless of size were about 65 per cent staminate and 35 per cent pistillate. These mixed populations refute the report that from each

holly berry the two large seeds will produce pistillate plants and the two small seeds, staminate plants. Furthermore, although there is a difference in the germination of seeds depending upon their size (Table I), once the seedlings are produced there seem to be no observable differences in their performance in the field. It is to be noted that none of the seedlings from the mixed lots of seeds had flowered by 1947, eight years after setting in the field. Plants at this time were ten or eleven years old, having been two or three years old at the time they were planted in the field.

That the age of the seedling at the time of setting in the field as well as the date of planting of the seeds had no effect on the sex ratios of the resulting plants is also shown in Table III.

The proportion of approximately one-third pistillate and two-thirds staminate plants grown from seeds represents more female seedlings than could be expected in natural plantings according to Coville (1). He stated (1, p. 5): "When seedlings are grown, it might be expected that the male and female trees would be present in about equal proportions, but this is not the case; seedlings from American and English holly seed are often in the proportion of 10 male to 1 female tree."

Less than 50 per cent of the plants flowered by 1947. A larger percentage could doubtless be brought into flower by extending the time of the experiment, but the mixed populations from each seed lot, combined with the crowding of the plants in the field, led to the termination of the investigation after the flowering season of 1947.

Although the ratio between the sexes of the total number of plants blooming has remained constant since 1944, it should be pointed out that final definite ratios could be determined only by extending the time of the experiment until all of the seedlings had produced flowers.

#### SUMMARY

Individual ripe holly berries were macerated and the seeds separated into large and small, two of each from each berry. These seeds were used for germination tests and some of the seedlings were grown to the flowering stage to determine a possible correlation between seed size and sex of the seedling. Germination tests were based on a total of 12,000 seeds, while 1255 seedlings were set in the field for sex determination.

It was found that the small seeds possessed approximately one-third of the germination capacity of the large seeds. However, the seedling populations from both lots were mixtures of pistillate and staminate plants in the proportion of approximately 1:2 thus showing no effect of seed size on sex determination.

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# AVAILABILITY OF PHOSPHORUS AND POTASSIUM OF SOME SOIL TYPES DEVOTED TO PASTURES IN NEW YORK

M. M. McCool

According to the New York Department of Agriculture and Markets (1) there were more than seven million acres of land devoted to pastures in New York State in 1937, a great deal of which is located on soil types which have medium to high lime requirements (2, 3, 4, 6, 7) and which produce low yields of inferior forage. Accordingly there are great possibilities for pasture land improvement in this State.

We were requested to make a study of the fertilizer needs of pastures which were grown on soil types acid in reaction. Laboratory and growth studies in the greenhouse were conducted. The project was terminated three years after it was inaugurated. Many samples of soil were taken from pastures which had not been limed, fertilized, or plowed for many years. Field observations were made each spring, summer, and autumn during the progress of the investigations.

## LITERATURE REVIEW

Spurway (9) early called attention to the fact that chemical tests do not afford information with respect to several physical factors which affect plant growth, and in addition the results derived from their use are not always in accord with plant growth either with or without the use of fertilizers. He expressed the belief that their chief value lies in determining whether a soil is unusually low in one or more elements which plants require for their development. Spurway (8) devised the Soiltex method by means of which the farmers of Michigan could ascertain the reaction of their soils. This did much to increase the acreage of alfalfa in that State.

Truog (11) sets the minimum limits of dilute acid extractable phosphorus for general farming under Wisconsin conditions at 75 pounds per acre in the plowed layer for the heavier soils and 50 pounds for the lighter sandy soils. According to him this limit should be raised to 150 pounds per acre or possibly higher still for garden and special crops.

Mehlich (5) as a result of his investigations with *Cunninghamella blakesleeana* reported that there was no definite relationship between the phosphorus response to crops in the field and the supply in soils as determined by the fungus method, except where it was very low on the one hand

and very high on the other. He also adopted standards by which to interpret results derived from the use of the *Aspergillus niger* method for measuring the availability of potassium in soils.

According to the interpretation of the results derived from the growth of *Aspergillus niger* in soils, an increase in the weight of the fungus mycelium which results from the addition of potassium of less than 20 per cent indicates a very low response, 21 to 40 per cent low, 41 to 65 per cent moderate, 66 per cent higher, and more than 90 per cent very high.

Additional methods together with results of a questionnaire on the use of methods of rapid tests in the United States were given by Thomas (10). Only five states reported no use of such tests. The total number of soil samples tested per state ranged from 50 to 500,000. It is notable that in Michigan, where the Soiltex method for testing the soil reaction was perfected and made available to farmers in that State for use on their farms at a very low cost, it was reported that 10,000 tests annually were made for farmers by county agricultural agents and others, whereas in South Carolina 500,000 samples were tested. Thomas also brings out that the reports for phosphorus indicate this element to be deficient in most soils.

Volk and Truog (12) used a large number of soils from widely separated areas in the perfection of a rapid chemical method for determining the readily available potash in soils. The extracting liquid adopted was normal ammonium acetate, pH value 6.8. They found the results derived from this method to correlate with those derived from field plot and *Aspergillus niger* methods.

Certain factors may be involved in the application of results derived from the use of quick tests with soil samples (11) notably the length of time which a plant occupies the soil, the nature and extent of its root system, and the variation in the ability of crops to remove one or more elements of plant food from the soil. In this connection grasses, notably Canadian blue (*Poa compressa* L.), orchard (*Dactylis glomerata* L.), Kentucky blue (*Poa pratensis* L.), chewings fescue (*Festuca rubra* L.), timothy (*Phleum pratense* L.), Pacys perennial rye grass (*Lolium perenne* L.), and red top (*Agrostis alba* L.) were grown in Gloucester and Podunk silt loam soils which were known to be deficient in available phosphorus.

#### MATERIALS AND METHODS

Soil samples were taken by the writer over widely separated areas to the depth to which the soils had been plowed, transported to the laboratory, and air dried for the laboratory studies. The bulk samples were screened and while in the fresh condition placed in glazed jars of two-gallon capacity. Sufficient amounts of precipitated calcium carbonate to bring the pH value to 6.4 were added and the fertilizer salts mixed with them. Pacys perennial rye grass seed was sown. After the rye grass was harvested the soils

were taken from some of the containers, and the grass roots removed. Soil from an area which carried bacteria that caused nodules to form on the roots of wild white clover (*Trifolium repens*) was mixed with them. The mixtures were returned to the containers and wild white clover seed sown in them.

The pH values of the samples were determined by means of a Beckmann glass electrode apparatus, the ratio of soil to water being 1 to 2.5, and the time of standing before the readings were taken amounted to ten minutes.

The readily available phosphorus was ascertained by means of the Truog (11) and the *Cunninghamella blakesleeana* (5) methods.

The *Aspergillus niger* (5) method was employed to determine the available potash.

## RESULTS

### LABORATORY STUDIES

The pH values of some of the soil samples collected are summarized in Table I. It is to be noted that the soils with the exceptions of Ontario and Dunkirk are quite acid.

TABLE I  
pH VALUES OF SOIL TYPES

Soil type	No. of items	Mean
Ontario	8	6.23
Dunkirk	6	5.66
Langford	15	5.28
Lordstown	14	5.17
Mardin	15	5.15
Culvers	10	5.01
Lackawanna	18	4.80
Gloucester	11	4.69
Dutchess	10	4.63

The amounts of dilute acid extractable or so called readily available phosphorus in the samples are given in Table II. With the exception of the Ontario soils, the tests show the types to be very low in readily available phosphorus.

The results derived from the use of the *Cunninghamella* method for measuring the availability of phosphorus in the soils comprise Table III. According to the standards suggested (5) the readily available supply is low in the Dunkirk group and medium in the remainder.

The data obtained from the *Aspergillus niger* method for determining the available potash are summarized in Table IV. According to this method of procedure the Dunkirk soils are very high, Langford medium, and the remainder high in readily available potash.



TABLE II  
DILUTE ACID EXTRACTABLE PHOSPHORUS IN SOIL TYPES

Soil type	No. of items	Mean p.p.m.
Ontario	7	42.0
Dunkirk	7	18.4
Langford	21	8.5
Gloucester	15	10.6
Dutchess	9	13.2
Mardin	20	8.5
Lackawanna	18	10.3
Lordstown	14	9.3
Culvers	12	6.7

### GREENHOUSE STUDIES

The results which were derived from growth tests with rye grass and white clover comprise Table V A and B. It is to be noted that nitrogen was supplied as nitrate of soda 100, phosphorus as superphosphate 200, and

TABLE III  
PHOSPHORUS AVAILABILITY BY CUNNINGHAMELLA METHOD

Soil type	No. of items	Mean growth diameter in mm.	Phosphorus supply
Langford	21	26.7	M
Lackawanna	35	21.2	M
Ontario	7	19.0	M
Lordstown	14	17.7	M
Culvers	12	17.2	M
Gloucester	15	17.1	M
Mardin	19	16.9	M
Dutchess	9	15.9	M
Dunkirk	7	9.6	L

TABLE IV  
POTASH AVAILABILITY BY ASPERGILLUS NIGER METHOD

Soil type	No. samples	Mean wt. in mg. of mycelium	Potash supply
Dunkirk	7	760	Very high
Dutchess	9	676	High
Culvers	12	633	High
Mardin	19	589	High
Lordstown	14	568	High
Lackawanna	18	565	High
Ontario	8	563	High
Gloucester	15	561	High
Langford	15	438	Medium

potassium in the form of potassium chloride 75 parts per million of soil, respectively.

According to the data in Table V A the growth of rye grass in all soils except the Volusia was enhanced by the addition of phosphorus. Thus the results are in accord with those derived by means of chemical tests, Table II, and also by the fungus growth, Table III. The application of lime to Langford soil furthermore resulted in greater yields of the rye grass.

TABLE V

A. FRESH WEIGHT IN GRAMS OF RYE GRASS WHEN GROWN IN DIFFERENT SOILS.  
AVERAGE OF FOUR REPLICATES

Cultural treatment	Soil types							
	Lackawanna	Lordstown	Mardin	Langford	Culvers	Dunkirk	Volusia	Ontario
O	4	22	9	31	14	14	14	35
P	12	44	15	41	55	36	16	53
PK	17	46	21	35	61	37	22	57
LPK	20	55	22	55	65	35	25	
NPK	67	90	73	47	95	92	75	71
LNPK	68	97	69	83	81	79	77	

B. FRESH WEIGHT IN GRAMS OF WILD WHITE CLOVER.  
AVERAGE OF FOUR REPLICATES

Soil types	Cultural treatment		
	L	LP	LPK
Lackawanna	4	37	16
Lordstown	34	60	71
Mardin	17	37	46
Langford	40	47	58
Culvers	2	14	9
Dunkirk	9	46	54
Volusia	20	51	44
Ontario	73	124	149

The data which comprise Table V B bring out also that the addition of superphosphate to the various soils resulted in greater yields of wild white clover. The yield of this crop was also augmented by the addition of potassium to the Ontario soil.

The results derived from studies with different grasses on the use of phosphorus on soils deficient in the readily available phosphorus comprise Table VI. The duration of the growth period was 35 days. The randomized arrangement of the blocks in the greenhouse was followed. Attention should be called to the fact that rye and orchard grasses yielded more in the cultures without the addition of phosphorus than did Kentucky blue, Canada blue, timothy, red top, and chewing fescue. Such differences

TABLE VI

DRY WEIGHT IN GRAMS OF GROWTH OF GRASSES, AVERAGE OF FOUR REPLICATES, IN GLOUCESTER AND PODUNK SOILS WITH AND WITHOUT THE ADDITION OF SUPERPHOSPHATE

Cultural treatment	Grass						
	Canada blue	Orchard	Kentucky blue	Chewings fescue	Timothy	Rye	Red top
Gloucester soil							
NKL	0.18	5	0.3	1	2	9	1
NPKL	8	17	9	10	18	16	17
Podunk soil							
NKL	0.25	4	0.4	2	3	9	2
NPKL	14	23	11	12	18	15	16

appear to be an important consideration in the most successful utilization of the soil and in the fertilizer practice to be followed.

#### SUMMARY AND CONCLUSIONS

Numerous samples of acid soils (pH 4.6 to 6.2) were taken from pastures in New York State and laboratory and greenhouse tests conducted to determine the availability of phosphorus and potassium therein.

Growth tests with rye grass brought out that with one exception there was a deficiency in the supply of available phosphorus in the soils taken. The yield of white clover in each soil was increased by the addition of superphosphate. According to the Truog method all soils except Ontario were very low in the amount of the readily available phosphorus and in addition the *Cunninghamella* method brought out that phosphorus was low in the Dunkirk group and medium in the remainder.

According to the *Aspergillus niger* method, the available potassium was very high in the Dunkirk and high in the remainder except the Langford in which it was medium.

Rye grass did not respond to the addition of potassium to the soils but the yield of white clover in Ontario soil was augmented by it.

Rye and orchard grasses yielded more in the cultures without the addition of phosphorus to soils low in readily available phosphorus than did other grasses utilized in the tests.

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## RESPIRATION RATE OF PLANT TISSUE UNDER CONDITIONS FOR THE PROGRESSIVE PARTIAL DEPLETION OF THE OXYGEN SUPPLY

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These experiments relate to the effect obtained on the respiration rate of plant tissue by continuously replenishing the oxygen consumed by an equal volume of nitrogen, thus bringing about a progressive partial depletion of the oxygen concentration from that of normal air (about 20.9 per cent by volume) to various lower percentages, such as to 15 per cent, 10 per cent, or even to lower levels.

The results have a bearing on previous respiration measurements made by a method in common use, that of absorbing in alkali the  $\text{CO}_2$  as fast as formed, and measuring the oxygen consumed by the change in pressure. Under these conditions the volume percentage of  $\text{O}_2$  changes continuously, and the question arises to what extent the rate of respiration has been affected by the change in the concentration of the  $\text{O}_2$  itself. They also have a bearing on experiments which have been carried out to determine the effect of added amounts of  $\text{CO}_2$  upon respiration rate. In such cases, also, the oxygen supply is being continuously depleted during the test, and doubt is introduced as to the true cause of any change in rate that results, i.e., whether the rate has been influenced mainly by the added amounts of  $\text{CO}_2$ , or by the decrease in the concentration of  $\text{O}_2$ .

The object of the present tests was to learn to what extent the  $\text{O}_2$  supply must be depleted before an effect upon the rate of respiration can be definitely observed, and to measure the extent of this effect at that stage of  $\text{O}_2$  depletion.

One might assume that an answer to such questions could be obtained by merely examining the literature relating to this problem. Such is not the case. The usual textbook statement of the situation is that the  $\text{O}_2$  concentration may deviate markedly from that of normal air without having any effect upon respiration rate (1, p. 351; 13, p. 277; or 14, p. 524). At the other extreme we find Blackman (2, 3) who states that " $\text{CO}_2$  production of the plant tissues yet examined varies with every alteration of the oxygen concentration in the environment." Whether this was intended to apply to the range from normal air content down to say 15 per cent  $\text{O}_2$  is not known, as no data by him in this region have been found. Platenius (15), also, emphasizes the influence of  $\text{O}_2$  on respiration rate by stating that "within the experimental range any decrease in the concentration of atmospheric oxygen produced a corresponding drop in the rate at which

oxygen was consumed." But his curves show only one measurement on one tissue (asparagus) in the region from approximately 12 per cent  $O_2$  to 20 per cent, and so furnish insufficient data on the shape of the curve in the region in which we are particularly interested at this time.

A quite different result was obtained by Mack (10), and by Mack and Livingston (11). Working with wheat seeds in the early stages of germination, they found that a reduction in the  $O_2$  concentration below that of normal air content not only did not bring about a retardation of the rate of  $CO_2$  production, but that it actually produced an increase in the rate. The percentage of  $O_2$  at which this first optimum occurred was different for different temperatures, but was from about 6.2 per cent  $O_2$  to 16 per cent  $O_2$ ; the extent of the increase in the rate at the lower concentration of  $O_2$  over that of the control at about 20 per cent  $O_2$  was from about 5 to 30 per cent. Reuhl (16) found a similar situation in the case of *Fagopyrum esculentum*. With seedlings in the later of the two stages of germination employed by her, reducing the  $O_2$  concentration below that of 20 per cent led to an increase in  $O_2$  consumption with an optimum rate at about 11 per cent  $O_2$ . However, with this same species at an earlier stage of germination, this increase was not observed, and furthermore the  $O_2$  concentration had to be reduced to about 5 per cent before there was any noticeable reduction in the rate of respiration. She worked with a number of other seeds, also, with results that depended upon the species, and the stage of germination at which the test was carried out. In most cases, however, it was necessary to reduce the  $O_2$  to values below 15 per cent to obtain definite reductions in respiration rate. Dolk and van Slogteren (7) allowed hyacinth bulbs to deplete the oxygen in the container progressively, and noted the oxygen concentration in the container at the time at which there occurred an evident reduction in the rate of  $O_2$  consumption; this occurred at an  $O_2$  concentration of about 14 to 15 per cent, or below. A new point of view was brought out by Chevillard, Hamon, Mayer, and Plantefol (5). They showed that in considering the effect of a reduced oxygen supply, a distinction should be made between the effect on the amount of  $CO_2$  produced and on the amount of  $O_2$  consumed. Although they obtained reductions in the rate of oxygen consumption by reducing the concentration of oxygen, e.g. to about 16 per cent  $O_2$  for *Hypnum triquetrum*, and to about 12 per cent  $O_2$  for potato tubers, they did not find a similar effect upon the amount of  $CO_2$  produced. There was no effect of oxygen concentration on the rate of  $CO_2$  production until the  $O_2$  concentration had been reduced to about 3 to 5 per cent (cress leaves presenting an exceptional case in showing a reduction in the rate of both  $O_2$  consumption and  $CO_2$  production when the  $O_2$  concentration had been reduced to about 9 per cent).

In the present experiments the controls consisted usually of four samples maintained at normal  $O_2$  content of 20.9 per cent by continuously

supplying  $O_2$  to the container of tissue as fast as the  $O_2$  was used up in respiration. For comparison there were four samples which received  $N_2$  to replace the  $O_2$  consumed, thereby the  $O_2$  content of the container being subjected to progressive depletion. Three objects were kept in mind: (a) to measure both  $O_2$  consumed and  $CO_2$  produced; (b) to use samples as large as proved to be feasible under the conditions of the experiment; (c) to let each experiment furnish its own measure of experimental error, so that a test of significance of observed differences could be made.

Since, from the results of previous experimenters, it was suspected that the differences, if any were obtained, would be small, use was made of the switch-back or reversal technique described by Brandt (4). By this procedure samples with which the  $O_2$  content was maintained during the first period were subjected to depletion of  $O_2$  during the second period, whereas samples starting out in the first period under conditions for depletion of  $O_2$  were switched to the other condition for the second period, receiving  $O_2$  continuously to maintain the normal  $O_2$  content. Four successive periods (three switch-backs) were used in these tests except for one tissue for which only two periods could be employed.

In general, the results show that with tubers of potato and Jerusalem artichoke, and with young seedlings of wheat, reducing the  $O_2$  concentration in the container to about 13 to 15 per cent reduced the  $O_2$  consumption by only about 4 to 6 per cent. The effect upon the  $CO_2$  production was even less, but a reduction in rate was established in experiments with three different tissues: with potato tubers when the  $O_2$  concentration was depleted to 2.4 per cent, with Jerusalem artichoke tubers with the  $O_2$  concentration brought down to 11.8 per cent, and with young wheat seedlings at about 10 to 11 per cent  $O_2$ . In these cases, also, the extent of the reduction in  $CO_2$  production was small, approximately 2 to 3 per cent, a difference that ordinarily would be regarded as within the limits of experimental error. However, the test of significance employed indicated a probability of less than 0.01 for differences as large as these in random sampling.

The results, then, do not confirm the older view that the  $O_2$  concentration must be reduced to very low values before an effect is had upon the respiration rate, or at least upon the rate of  $O_2$  consumption. A retarding effect was found, even though the extent of the effect was not large. The results likewise cast doubt on the generality of the newer view that any alteration of the  $O_2$  below that of normal air results in a retardation of the rate of respiration. In these tests, the tissues were not found to be very sensitive toward small changes in  $O_2$  concentration, at least in the range just below the concentration of normal air. Of course, the duration of the exposure of the tissue to the different  $O_2$  concentrations may prove to be a factor in future tests.



## MATERIAL AND METHODS

**Tissue.** The tubers of potato (*Solanum tuberosum* L. var. Irish Cobbler) and Jerusalem artichoke (*Helianthus tuberosus* L.) were grown in the Institute garden. They were removed from storage at 5° C. and allowed to remain in burlap bags for one week in the experimental room at 20° C. previous to the start of the respiration tests. The potato tuber samples consisted of either 67 tubers weighing 2000 g., or of 80 tubers weighing 2250 g., in each lot. The respiration of the artichoke tubers was found to be much higher, however, so that the samples consisted either of 23 tubers weighing 400 g., or of 27 tubers weighing 625 g. The leaves of *Pelargonium domesticum* Bailey were approximately fully grown with long petioles. These petioles were then placed in water in small vials, 9 mm. diam. × 75 mm., three leaves in each vial, the petioles so chosen that pressure was needed to force them into the vial. This allowed the leaves to be put into and taken out of the desiccators without becoming detached from the vials. The water in the vial was sufficient for maintaining the leaves in a turgid condition during the respiration test, approximately 60 hours. Each sample consisted of 48 leaves, fresh weight approximately 112 g., dry weight approximately 7 g. The wheat (*Triticum aestivum* L.) seeds were purchased from a seedsman, and were of the variety Leap's Prolific. About 95 per cent germination was obtained. The seeds were weighed into lots of 25 g. air dry weight, each lot being tied in a cheesecloth bag. Previous to an experiment the bags of seeds were soaked for 22 to 24 hours in water at 20° C. Then the bags were removed, one by one, and shaken forcibly to dislodge the excess water, and were dipped into a suspension of "Wettable Sperguson" containing 1 g. of this chemical in 100 cc. of water. The bags were again shaken to dislodge the excess liquid, and each bag was placed on a wire screen tray 11 cm. × 11 cm. × 1 cm.; the cloth was spread out over the bottom of the tray, and the seeds were distributed into an even layer with a spatula. The trays were piled on top of one another, criss-cross fashion, in the respiration chamber. With experiments starting at an early stage of germination the seed samples were placed in the respiration vessels at once after the preliminary soaking, but when a test was to be made at a later stage of germination the piles of screens containing seeds were placed in wooden flats with moist burlap in the bottoms, covered with inverted flats, and stored at 20° C. Under these moist conditions germination proceeded rapidly. At no time was any contamination by mold growth observed in these tests.

**Apparatus.** As containers for the tissue during a respiration test, glass desiccators of approximately 6-liter capacity were used. First, 100 cc. of a NaOH solution containing either 65 g. or 80 g. of NaOH per liter of water (depending upon the amount of CO<sub>2</sub> expected) were placed in a porcelain evaporating dish 14 cm. in diameter in the bottom of the desiccator, then

the tissue was distributed on the shelf, and after the well-greased cover was replaced, the desiccator was immersed in a can of water, being held in place by sandbags. The desiccators were of the bowl-type, i.e., without a conical constriction at the base. The shelf rested on lugs near the bottom. The area of the alkaline solution used for absorbing  $\text{CO}_2$  was approximately 95 sq. cm., and its surface was about 5 cm. below the lowest layer of respiring tissue. On numerous occasions a sample of the air in the container was taken at the end of a test, and analyzed for  $\text{CO}_2$ , using a 100 cc. gas burette with 0.2 cc. calibrations. In no case was a measurable percentage of  $\text{CO}_2$  found in the air surrounding the tissue at the end of an experiment.

Further description can be followed more conveniently by reference to Figure 1 A, B, C. The apparatus used for measuring the oxygen consumed and carbon dioxide produced was a modification of that first described by Magness and Diehl (12), and subsequently improved by various experimenters, among them being Haller and Rose (8), and Whiteman and Schomer (18). The procedure finally adopted in these tests was as follows. The capillary tube siphon, Figure 1 A, produces a constant slow dripping of water from the water reservoir into the funnel. This funnel has an over-flow tube in the center, and this establishes a constant water level in the funnel. The length and bore of the capillary tube, Figure 1 Aa, establishes the rate of dropping into the funnel. During many months of operation this siphon has never become clogged or failed to maintain the water level at all times. The over-flow from the funnel collects in the metal can, Figure 1 B, and occasionally this water is drawn off at the spigot and is returned to the reservoir. The glass cylinder, Figure 1 Bb (throughout this paper referred to as the "side-tube"), contains the oxygen which is to replace the oxygen used up by the respiration in the desiccator containing the plant tissue. By suction at Figure 1 Bc, the water siphon is established between the funnel and the glass tubing which passes up through the center of the side-tube, b. The side-tube is lowered slightly so that water runs into it until the zero mark at the bottom of the side-tube is reached. Then the side-tube is raised until the water at the tip of the inner glass tube is even with the water level in the funnel. The water at the tip of the inner glass tubing will then show a flat, not rounded, meniscus. A jaw-type pinch clamp placed temporarily on the rubber tubing at about the place marked, Figure 1 Bd, will facilitate these operations in adjusting the water to the zero mark, and the water level in the tubing to that in the funnel. Also, the pinch clamp is useful when the side-tube is to be filled up with oxygen for the succeeding test; but the clamp must be removed when the respiration test is started. The dark-appearing object in the funnel is a rubber stopper with center hole for the over-flow tube and acts as a weight to hold down the end-tubes of the siphon. The small Y-tubes at the top of the siphon are needed for the collection of any air bubbles that enter the siphon-

tubes. Connection is made from the side-tube to the desiccator containing the plant tissue by means of the capillary tube, Figure 1 Be.

Figure 1 C is a set-up of four lots of tissue running simultaneously in a constant temperature room at 20° C. and obtaining a constant water supply and constant water level from one over-flow funnel and one water reservoir. The desiccators are immersed in water in the galvanized iron cans and are weighted down with sandbags. Another group of four units at the left (not shown) was employed in the tests.

The operation during a respiration test is as follows. Oxygen is consumed in respiration and the CO<sub>2</sub> which escapes from the tissue is absorbed by the alkali in the bottom of the desiccator. This reduction in pressure is communicated through the capillary tube, e, to the oxygen side-tube, b; water then flows through the siphon from the funnel and an equal quantity of oxygen is pushed from the oxygen side-tube into the desiccator. At the end of a test, or at intervals during it if desired, the volume of water that has been siphoned over is read from the calibration marks on the side-tube, b. Since the volumes of the desiccator, tissue, the absorbing alkali and its container, and of the side-tube (b) are known, the net volume of gas in the apparatus subject to change in pressure can be found. The barometer reading at the start of the test permits computing the volume of gas at the start, expressed at say 0° C. and 760 mm. At the end of a test the volume of water in the calibrated side-tube is subtracted from the total volume of the side-tube, the difference being the volume of oxygen remaining unused at the top of the side-tube. This volume plus the net volume of the desiccator shows the volume of gas subject to pressure change at the end of a test. The barometric reading at the end of the test permits converting this volume to the standard condition of 0° C. and 760 mm. The difference between these two volumes, at the start and the end of a test, shows the volume of oxygen at 0° C. and 760 mm. consumed during the respiration period. The alkali in the bottom of the desiccator is then rinsed into a volumetric flask, a quantity of saturated BaCl<sub>2</sub> solution sufficient to precipitate the CO<sub>3</sub> is added, and water is added to the mark. Titration of this solution with 0.25 N HCl shows the amount of unused alkali, and the difference between this value and a blank obtained by the use of an equal quantity of the alkali solution subjected to the same procedure permits computing the volume of CO<sub>2</sub> formed during the test, this value also being expressed at 0° C. and 760 mm.

The description so far given relates to replacing the oxygen consumed in respiration with an equal quantity of oxygen from the oxygen-supply tube. But in these experiments when the effect of progressive depletion of the oxygen supply was to be measured the side-tube was filled not with O<sub>2</sub> but with N<sub>2</sub>, so that as O<sub>2</sub> was used up in respiration it was replaced by N<sub>2</sub>.

It is necessary to consider the question whether a correction needs to

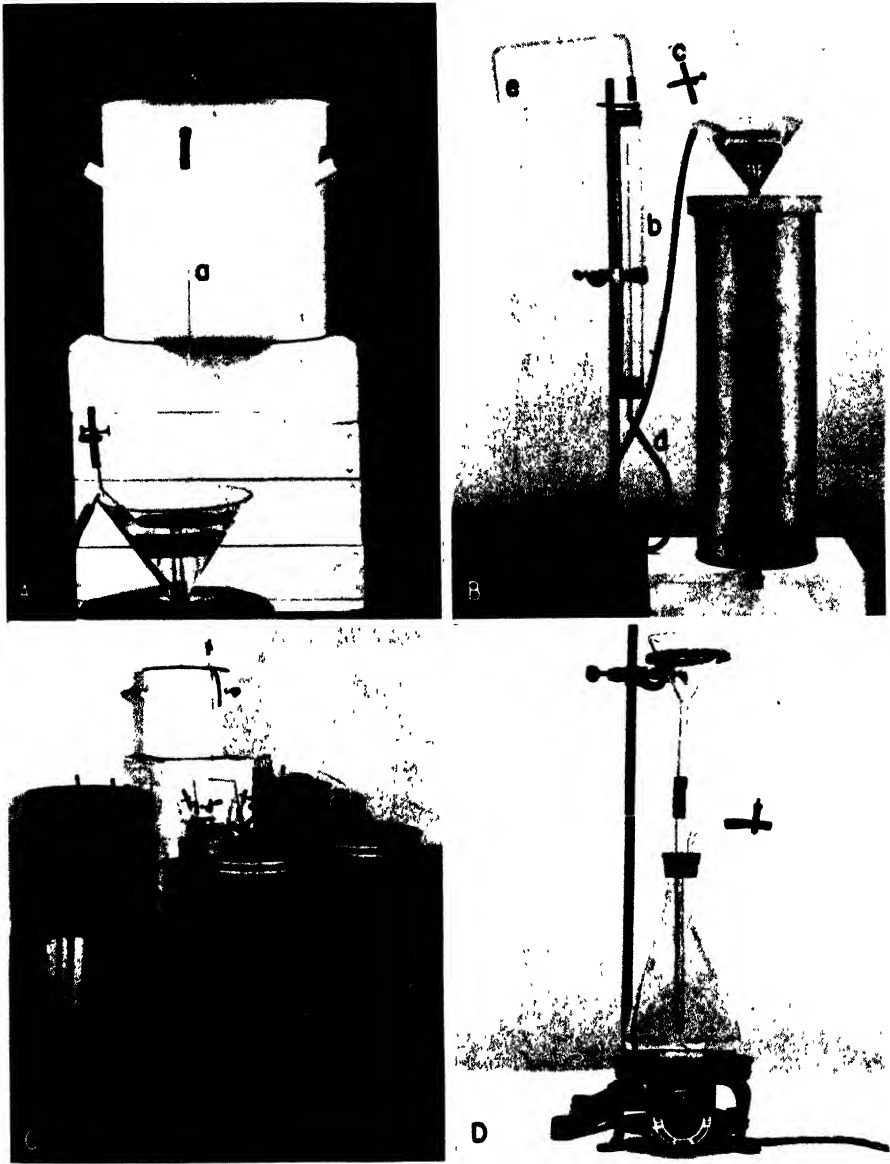


FIGURE 1. A, B, C. Apparatus for measuring  $O_2$  consumed and  $CO_2$  produced. A, method of maintaining a constant water level; B, method of supplying  $O_2$  (or  $N_2$ ) to tissue and measuring volume of water siphoned over; C, a set-up of four lots of tissue in desiccators immersed in cans of water in a constant temperature room; D, apparatus for boiling off dissolved gases from water.

be made for the solubility of  $O_2$  or  $N_2$  in the water in the side-tubes. As the water enters from the funnel it flows as a thin film down the inner tube and has a good opportunity to dissolve  $O_2$  (or  $N_2$ ), as the case may be, and this would tend to overestimate, or underestimate, the amount of  $O_2$  consumed. Now, the water entering the funnel was tap water already containing some dissolved air, and no tables were found which would allow computing the amount of  $O_2$  or  $N_2$  which would be taken up or released on contact with these gases under the conditions of these experiments. Therefore, it seemed that an estimate of the solubility factor could best be made by estimating the  $O_2$  and  $N_2$  content of the water in the funnel and of the water that had accumulated in the side-tubes containing either  $O_2$  or  $N_2$  at the end of a respiration test. This was done by boiling off the dissolved gases and analyzing them by gas-analysis methods. The apparatus used for forcing the dissolved gases out of solution is shown in Figure 1 D. The water was poured into the Erlenmeyer flask to the brim, and the rubber stopper was forced in, care being taken that no air was trapped either in the flask or in the outlet tube. The funnel allowed for the expansion during heating and for transferring the liberated gas through the tube to the gas-analysis apparatus. The curvature of the tube at the bottom prevented loss of air from the bottom of the flask upward in the tube. Adjustment of the heat from the hot plate allowed boiling to proceed smoothly. About one-half hour of heating was sufficient to drive off all of the gas that could be obtained in the way, as shown by removing the gas at intervals and noting roughly when further contributions of gas volumes became small. The water entering from the funnel was found to contain approximately 4.5 cc. of  $O_2$  and 10.1 cc. of  $N_2$  (at  $0^\circ$ , 760 mm.) per liter; the water in the  $O_2$  side-tubes at the end of a test contained approximately 15.6 cc. of  $O_2$  and 2.8 cc. of  $N_2$ ; the corresponding values for the water in the  $N_2$  side-tubes at the end were: 1.0 cc. of  $O_2$  and 10.9 cc. of  $N_2$ . The computations for the corrections needed for the experiments when  $O_2$  was supplied from the side-tubes are:

$O_2$ in $H_2O$ in side-tube at end	= 15.6 cc.
" " " that entered	= <u>4.5</u>
$O_2$ dissolved	= 11.1 cc. (to be deducted from reading)
$N_2$ in $H_2O$ that entered	= 10.1 cc.
" " " in side-tube at end	= <u>2.8</u>
$N_2$ released into side-tube	= 7.3 cc. (to be added to reading)
Net effect = 11.1 - 7.3 = 3.8 cc. to be deducted for each 1000 cc. of $H_2O$ in $O_2$ side-tube at end.	

However, the amount of  $H_2O$  collected in one of the side-tubes in any single test was never as much as 700 cc., and usually was about 200 to 400

cc., so that the correction to be applied was less than 0.5 per cent of the amount of the reading.

The corresponding values for the N<sub>2</sub> side-tubes were:

N<sub>2</sub> in H<sub>2</sub>O in side-tube at end = 10.9 cc.

" " " that entered = 10.1

N<sub>2</sub> dissolved = 0.8 cc. (to be deducted from reading)

O<sub>2</sub> in H<sub>2</sub>O that entered = 4.5 cc.

" " " in side-tube at end = 1.0

O<sub>2</sub> released into side-tube = 3.5 (to be added to reading)

Net effect = 3.5 - 0.8 = 2.7 cc. to be added for each 1000 cc. of H<sub>2</sub>O in N<sub>2</sub> side-tube at end.

The corrections for solubility of gases in either O<sub>2</sub> or N<sub>2</sub> side-tubes were so small as to seem not worth making. Consequently no correction for solubility of gases was made in any of these experiments.

## RESULTS

### POTATO TUBERS

Table I shows the result of an experiment with potato tubers in which the oxygen content of the air surrounding the tubers was maintained at approximately 20.9 per cent O<sub>2</sub> in one set of four samples by supplying O<sub>2</sub> to the tubers as fast as it was consumed, while with another set of four samples the O<sub>2</sub> consumed was replaced continuously with N<sub>2</sub>. In this way the O<sub>2</sub> in the desiccators in the second group was depleted finally to approximately 13.3 per cent O<sub>2</sub>.

The "reversal" or "switch-back" method described by Brandt (4) was used. There were four successive periods of 25 hours each. In the "A" group of four lots of tubers O<sub>2</sub> was supplied by the O<sub>2</sub> side-tubes so that the O<sub>2</sub> content was maintained for the first period; in the second period of 25 hours N<sub>2</sub> was supplied as fast as O<sub>2</sub> was used in respiration, the O<sub>2</sub> in the container becoming partially depleted (to approximately 13.3 per cent O<sub>2</sub>); for the third period of 25 hours these lots were switched back to O<sub>2</sub> side-tubes, and finally for the fourth period again returned to N<sub>2</sub> side-tubes. The four samples marked "Group B" in Table I received the replenishing gases in the reverse order to that described for Group A, i.e., receiving N<sub>2</sub> as the replenishing gas for the first period of 25 hours, O<sub>2</sub> in the second period, N<sub>2</sub> in the third period, and O<sub>2</sub> in the final period.

The values shown in the body of Table I are the volumes of O<sub>2</sub> consumed and CO<sub>2</sub> produced by each of the eight samples of tubers in each of the four successive periods. For example the lot in the first line consumed 264 cc. of O<sub>2</sub> in the first period when its O<sub>2</sub> consumption was replenished with O<sub>2</sub>, 261 cc. in the second period when the O<sub>2</sub> consumed was replenished

with  $N_2$  ( $O_2$  partially depleted), 271 cc. in the third period when it was returned to an  $O_2$  side-tube, and 258 cc. in the final period when it was returned to  $N_2$ .

The method of computing the significance of differences observed in a "reversal" or "switch-back" experiment is described in detail by Brandt (4), and only enough of it will be repeated here to assist the reader in following the computations in this paper. For example, the value  $-36$  in line 1, column 7, Table I, is obtained as follows:  $-264 + 3(261) - 3(271) + 258 = -36$ . Alternately, it may be obtained by computing the successive differences between the four measurements as follows:

$$\begin{array}{rclcl}
 264 & & & & \\
 & - 3 & & & \\
 261 & & + 13 & & \\
 & + 10 & \cdot & & - 36 \\
 271 & & - 23 & & \\
 & - 13 & & & \\
 258 & & & & 
 \end{array}$$

These two methods seem quite different, but they are in reality identical, as can be seen by the following scheme, assigning the letters A, B, C, D to represent the four values:

$$\begin{array}{lcl}
 A & & \\
 B - A & & \\
 B & C - B - B + A & \\
 C - B & & D - C - C + B - C + B + B - A \\
 C & D - C - C + B & \\
 D - C & & \\
 D & & 
 \end{array}$$

and when the third difference is put in the form  $-A + 3B - 3C + D$  it is seen to indicate the operation shown above with the values given in line 1, columns 3 to 6, Table I.

The "sum of squares" for Group A, 489, is obtained as follows:

$$(-36)^2 + (-55)^2 + (-27)^2 + (-29)^2 - \frac{(-147)^2}{4} = 489$$

(or more conveniently usually by operating from an assumed mean of the four values). In the "Notes" at the bottom of Table I are shown the methods of computing "s" (the standard deviation) and "t" (which shows the ratio of the difference between the means to the standard error of their differences).

For the data relating to the  $O_2$  consumed, the "t" value 7.94 with 6 degrees of freedom (3 D. F. for each set of 4 samples) is highly significant,

and shows that the partial depletion of the O<sub>2</sub> in this experiment decreased the rate of oxygen consumption. The amount of this decrease, however, was found to be quite small, the total O<sub>2</sub> consumption of the lots with partially depleted O<sub>2</sub> supply being only 4.2 per cent below that of the control lots with O<sub>2</sub> maintained.

The data for CO<sub>2</sub> produced (columns 8 to 11, Table I), however, do not

TABLE I

EFFECT OF PARTIAL DEPLETION OF OXYGEN ON RESPIRATION RATE OF POTATO TUBERS  
(O<sub>2</sub> CONTENT DEPLETED FROM 20.9 PER CENT O<sub>2</sub> TO AN AVERAGE  
OF 13.3 PER CENT O<sub>2</sub> IN 25 HOURS)

Group	Order for replenishing O <sub>2</sub> used up	c.c. O <sub>2</sub> (0°, 760) consumed (by 2 kg.) in period No.				Diff.	Cc. CO <sub>2</sub> (0°, 760) produced (by 2 kg.) in period No.				D'ff.
		1	2	3	4		1	2	3	4	
A	O <sub>2</sub> , N <sub>2</sub> , O <sub>2</sub> , N <sub>2</sub> in the four successive periods	264	261	271	258	- 36	271	275	264	258	+20
		260	247	260	244	- 55	277	272	275	273	-13
		284	275	278	266	- 27	295	285	281	281	- 2
		264	260	264	247	- 29	277	281	267	271	+36
		Sum				-147	Sum				+41
		Mean				- 36.75	Mean				+10.25
B	N <sub>2</sub> , O <sub>2</sub> , N <sub>2</sub> , O <sub>2</sub> in the four successive periods	Sum of squares				489	Sum of squares				1449
		262	280	268	275	+ 49	281	274	275	275	- 9
		258	275	270	280	+ 37	288	284	283	281	- 4
		273	282	277	283	+ 25	284	287	287	293	+ 9
		264	284	269	280	+ 61	303	293	290	287	- 7
		Sum				+172	Sum				-11
		Mean				43.00	Mean				- 2.75
		Sum of squares				720	Sum of squares				197

Notes:  $s = \sqrt{\frac{489 + 720}{3 + 3}} = 14.2$ ; D.F. = 3 + 3 = 6

$$t = \frac{43 - (-36.75)}{14.2} \sqrt{\frac{(4)(4)}{4 + 4}} = 7.94; \text{Prob.} < .01$$

Total O<sub>2</sub> consumed by lots with O<sub>2</sub> maintained = 4384 cc.  
Total O<sub>2</sub> consumed by lots with O<sub>2</sub> depleted = 4199 cc.  
Decrease = 185 cc. = 4.2% below control rate

$s = 16.57$ ; D.F. = 6

$$t = 1.110; \text{Prob.} = 0.3$$

Total, O<sub>2</sub> maintained = 4481 cc. CO<sub>2</sub>  
Total, O<sub>2</sub> depleted = 4487 cc. CO<sub>2</sub>  
Increase = 6 cc. = 0.13%

show significant differences between the lots with O<sub>2</sub> maintained and O<sub>2</sub> depleted.

In a succeeding experiment with potato tubers, in view of the rather small reduction in the rate of O<sub>2</sub> consumption by the lots with partially depleted O<sub>2</sub> supply, and of the failure to find any differences at all in the rate of CO<sub>2</sub> production, a larger quantity of tubers in each lot was employed, and the experiment was allowed to continue longer, until, in fact, nearly all of the O<sub>2</sub> in the containers was used up by the lots in which a pro-



gressive depletion of the O<sub>2</sub> was permitted to occur. The results are shown in Table II.

In this experiment, even the rate of CO<sub>2</sub> production was decreased by the depletion of the O<sub>2</sub>. The amount of the reduction in rate was small, it is true, being only 1.73 per cent below that of the control; but the "t" value

TABLE II

EFFECT OF DRASTIC DEPLETION OF OXYGEN ON RESPIRATION RATE OF POTATO TUBERS  
(O<sub>2</sub> CONTENT DEPLETED FROM 20.9 PER CENT O<sub>2</sub> TO AN AVERAGE  
OF 2.4 PER CENT O<sub>2</sub> IN 40.5 HOURS)

Group	Order for replenishing O <sub>2</sub> used up	Cc. O <sub>2</sub> (0°, 760) consumed (by 2.25 kg.) in period No.				Diff.	Cc. CO <sub>2</sub> (0°, 760) produced (by 2.25 kg.) in period No.				Diff.
		1	2	3	4		1	2	3	4	
A	O <sub>2</sub> , N <sub>2</sub> , O <sub>2</sub> , N <sub>2</sub> in the four successive periods	541	494	640	596	- 383	498	529	602	631	- 86
		546	520	654	615	- 333	544	561	634	664	- 99
		593	523	703	620	- 513	555	578	662	675	- 132
		596	527	683	595	- 469	549	574	644	677	- 82
		Sum				- 1698	Sum				- 399
		Mean				- 424.5	Mean				- 99.75
B	N <sub>2</sub> , O <sub>2</sub> , N <sub>2</sub> , O <sub>2</sub> in the four successive periods	Sum of squares				19907	Sum of squares				1545
		489	574	551	684	+ 264	495	535	588	634	- 20
		489	572	545	662	+ 254	519	558	588	635	+ 26
		493	619	580	727	+ 351	542	582	624	687	+ 19
		481	579	548	683	+ 295	515	552	601	654	- 8
		Sum				+ 1164	Sum				+ 17
		Mean				+ 291	Mean				+ 4.25
		Sum of squares				5714	Sum of squares				1429

Notes:  $s = \sqrt{\frac{19907 + 5714}{3 + 3}} = 65.34$ ; D.F. = 3 + 3 = 6

$t = \frac{+291 - (-424.5)}{65.34} \sqrt{\frac{(4)(4)}{4 + 4}} = 15.48$ ; Prob. < .01

Total O<sub>2</sub> consumed by lots with O<sub>2</sub> maintained = 10056 cc.  
 Total O<sub>2</sub> consumed by lots with O<sub>2</sub> depleted = 8666 cc.  
 Decrease = 1390 cc. = 13.8% below control rate

$s = 22.26$ ; D.F. = 6

$t = 6.61$ ; Prob. < .01

Total O<sub>2</sub> maintained = 9525 cc. CO<sub>2</sub>  
 Total O<sub>2</sub> depleted = 9361 cc. CO<sub>2</sub>  
 Decrease = 164 cc. = 1.73%

for this test was 6.61 with 6 D. F., while the required value for a probability of 0.01 or less is 3.707.

#### ARTICHOKE TUBERS

An experiment employing the "reversal" or "switch-back" procedure with Jerusalem artichoke tubers is shown in Table III. In this case the O<sub>2</sub> with the lots undergoing progressive depletion of the O<sub>2</sub> supply was reduced to 14.8 per cent O<sub>2</sub> in a period of 24 hours. As shown by the data

at the bottom of Table III, this extent of O<sub>2</sub> depletion decreased the oxygen consumption by 5.4 per cent below the rate of the controls with O<sub>2</sub> concentration continuously maintained. The "t" value was 3.65, corresponding to a probability of slightly more than 0.01. Again, however, no difference, or at least only a small and non-significant one, was found in the rate of

TABLE III  
EFFECT OF PARTIAL DEPLETION OF OXYGEN ON RESPIRATION RATE OF  
JERUSALEM ARTICHOKE TUBERS  
(O<sub>2</sub> CONTENT DEPLETED FROM 20.9 PER CENT O<sub>2</sub> TO AN AVERAGE OF  
14.8 PER CENT O<sub>2</sub> IN 24 HOURS)

Group	Order for replenishing O <sub>2</sub> used up	Cc. O <sub>2</sub> (0°, 760) consumed (by 0.4 kg.) in period No.				Diff.	Cc. CO <sub>2</sub> (0°, 760) produced (by 0.4 kg.) in period No.				Diff.
		1	2	3	4		1	2	3	4	
A	O <sub>2</sub> , N <sub>2</sub> , O <sub>2</sub> , N <sub>2</sub> in the four successive periods	321	280	293	308	- 52	294	280	293	322	- 11
		306	291	289	311	+ 11	294	288	286	312	+24
		286	267	283	307	- 27	273	268	274	307	+16
		296	255	264	277	- 46	287	269	261	294	+31
		Sum				- 114	Sum				+60
		Mean				- 28.5	Mean				+15
B	N <sub>2</sub> , O <sub>2</sub> , N <sub>2</sub> , O <sub>2</sub> in the four successive periods	Sum of squares				2421	Sum of squares				1014
		237	243	238	273	+ 51	231	229	246	273	- 9
		254	263	272	292	+ 11	268	263	283	307	- 21
		241	258	235	276	+104	256	243	245	275	+13
		274	325	317	344	+ 94	294	315	325	343	+19
		Sum				+260	Sum				+ 2
		Mean				+ 65.0	Mean				+ 0.5
		Sum of squares				5474	Sum of squares				1051

Notes:  $s = \sqrt{\frac{2421 + 5474}{3 + 3}} = 36.27$ ; D.F. = 3 + 3 = 6

$$t = \frac{+65 - (-28.5)}{36.27} \sqrt{\frac{(4)(4)}{4 + 4}} = 3.65; \text{Prob} = .011$$

Total O<sub>2</sub> consumed by lots with O<sub>2</sub> maintained = 4612 cc.  
Total O<sub>2</sub> consumed by lots with O<sub>2</sub> depleted = 4364 cc.  
Decrease = 248 cc. = 5.4% below control rate

$s = 18.55$  D.F. = 6

$t = 1.11$  Prob. = .35

Total, O<sub>2</sub> maintained = 4510 cc. CO<sub>2</sub>  
Total, O<sub>2</sub> depleted = 4488 cc. CO<sub>2</sub>  
Decrease = 22 cc. = 0.5%

CO<sub>2</sub> production (Table III, columns 8 to 12, and at the bottom of the table under these columns).

In a second experiment with Jerusalem artichoke tubers, the quantity of tubers in a sample was increased, and the depletion of O<sub>2</sub> in the lots receiving N<sub>2</sub> as replenishment for the O<sub>2</sub> consumed was allowed to continue until a final value of 11.8 per cent O<sub>2</sub> was reached. The results are shown in Table IV. As seen from the "t" values at the bottom of the table, partial

progressive depletion of the O<sub>2</sub> supply brought about a reduction in the rate of respiration, with respect to both oxygen consumption and CO<sub>2</sub> production. In the case of oxygen consumed the value for lots with O<sub>2</sub> partially depleted was 5.6 per cent below that of the control lots. The CO<sub>2</sub> produced by the lots with O<sub>2</sub> partially depleted was only 1.55 per cent below that of

TABLE IV  
EFFECT OF PARTIAL DEPLETION OF OXYGEN ON RESPIRATION RATE OF  
JERUSALEM ARTICHOKE TUBERS  
(O<sub>2</sub> CONTENT DEPLETED FROM 20.9 PER CENT O<sub>2</sub> TO AN AVERAGE OF  
11.8 PER CENT O<sub>2</sub> IN 16.5 HOURS)

Group	Order for replenishing O <sub>2</sub> used up	Cc. O <sub>2</sub> (0°, 760) consumed (by 0.625 kg.) in period No.				Diff.	Cc. CO <sub>2</sub> (0°, 760) produced (by 0.625 kg.) in period No.				Diff.
		1	2	3	4		1	2	3	4	
A	O <sub>2</sub> , N <sub>2</sub> , O <sub>2</sub> , N <sub>2</sub> in the four successive periods	363	344	393	359	-151	360	355	360	371	-31
		366	341	375	356	-112	359	358	368	379	-10
		425	392	427	374	-156	422	413	421	399	-47
		433	402	415	401	-71	438	415	416	420	-12
		Sum				-490	Sum				-100
		Mean				-122.5	Mean				-25
B	N <sub>2</sub> , O <sub>2</sub> , N <sub>2</sub> , O <sub>2</sub> in the four successive periods	Sum of squares				4697	Sum of squares				914
		358	372	367	385	+42	365	366	366	385	+20
		374	385	379	392	+36	381	390	388	395	+20
		371	386	363	380	+78	378	383	365	385	+61
		422	456	407	413	+138	441	455	428	419	+59
		Sum				+294	Sum				+160
		Mean				+73.5	Mean				+40
		Sum of squares				6579	Sum of squares				1602

Notes:  $s = \sqrt{\frac{4697+6579}{3+3}} = 43.34$ ; D.F. = 3+3 = 6

$t = \frac{+73.5 - (-122.5)}{43.34} \sqrt{\frac{(4)(4)}{4+4}} = 6.20$ ; Prob. = <.01

Total O<sub>2</sub> consumed by lots with O<sub>2</sub> maintained = 6366 cc.  
 Total O<sub>2</sub> consumed by lots with O<sub>2</sub> depleted = 6010 cc.  
 Decrease = 356 cc. = 5.6% below control rate

$s = 20.47$ ; D.F. = 6

$t = 4.49$ ; Prob. = <.01

Total, O<sub>2</sub> maintained = 6329 cc. CO<sub>2</sub>  
 Total, O<sub>2</sub> depleted = 6231 cc. CO<sub>2</sub>  
 Decrease = 98 cc. = 1.55%

the corresponding controls with O<sub>2</sub> maintained, but the "t" value found was 4.49, which indicates a highly significant difference in the rates.

#### PELAGONIUM LEAVES

The results of a test with pelargonium leaves are shown in Table V. In attempting to apply the "reversal" or "switch-back" procedure to this material it was found that only one reversal (two consecutive periods)

could be used, since a period of 29.5 hours was necessary to bring the O<sub>2</sub> concentration down even to 16.0 per cent with the lots undergoing progressive depletion of O<sub>2</sub> supply. This gave a total period of about 60 hours to complete one reversal, which was all that detached leaves with petioles in

TABLE V

EFFECT OF PARTIAL DEPLETION OF OXYGEN ON RESPIRATION OF PELLARGONIUM LEAVES  
(O<sub>2</sub> CONTENT DEPLETED FROM 20.9 PER CENT O<sub>2</sub> TO AN AVERAGE OF  
16.0 PER CENT O<sub>2</sub> IN 29.5 HOURS)

Group	Order for replenishing O <sub>2</sub> used up	Cc. O <sub>2</sub> (o°, 760) consumed by 112 g. fresh weight of leaves in period No.		Diff.	Cc. CO <sub>2</sub> (o°, 760) produced by 112 g. fresh weight of leaves in period No.		Diff.
		1	2		1	2	
A	O <sub>2</sub> , N <sub>2</sub> in the two successive periods	233	294	— 61	228	255	— 27
		221	264	— 43	218	251	— 33
		218	246	— 28	214	234	— 20
		236	249	— 13	231	245	— 14
		Sum		— 145	Sum		— 94
		Mean		— 36.25	Mean		— 23.5
Sum of squares		1267	Sum of squares		205		
B	N <sub>2</sub> , O <sub>2</sub> in the two successive periods	229	285	— 56	231	245	— 14
		228	281	— 53	227	271	— 44
		221	252	— 31	221	242	— 21
		217	273	— 56	221	259	— 38
		Sum		— 196	Sum		— 117
		Mean		— 49.0	Mean		— 29.25
Sum of squares		438	Sum of squares		595		

Notes:  $s = \sqrt{\frac{1267+438}{3+3}} = 16.86$ ; D.F. = 3+3=6

$$t = \frac{-36.25 - (-49.0)}{16.86} \sqrt{\frac{(4)(4)}{4+4}} = 1.060; \text{Prob.} = .33$$

Total O<sub>2</sub> consumed by lots with O<sub>2</sub> maintained = 1999 cc.  
Total O<sub>2</sub> consumed by lots with O<sub>2</sub> depleted = 1948 cc.  
Decrease = 51 cc. = 2.5% below control rate

$s = 11.54$ ; D.F. = 6

$$t = 0.704; \text{Prob.} = .51$$

Total, O<sub>2</sub> maintained = 1908 cc. CO<sub>2</sub>  
Total, O<sub>2</sub> depleted = 1885 cc. CO<sub>2</sub>  
Decrease = 23 cc. = 1.2%

vials of water could endure without unfavorable effects upon their condition. It was not found feasible to use a larger quantity of leaves to increase the rate of O<sub>2</sub> depletion on account of lack of space in the desiccator for a larger number of leaves. However, the leaves were in fairly good condition at the end of the two periods, turgid and with only a few leaves showing yellowing. The results are shown in Table V. Although both with O<sub>2</sub>-consumed and CO<sub>2</sub>-produced the O<sub>2</sub>-depleted lots showed slightly lower values these differences are not significant by the "t" test. The situation

with regard to the pelargonium leaves is, then, inconclusive. It was decided not to continue the tests on such material until the experimental procedure could be revised so as to fit larger quantities of leaves into a smaller space, and thus increase the rate and extent of  $O_2$  depletion.

TABLE VI  
EFFECT OF PARTIAL DEPLETION OF  $O_2$  CONCENTRATION UPON RESPIRATION  
RATE OF GERMINATING WHEAT SEED

Exp. No. and % O <sub>2</sub> at end	Cc. (at 0°, 760 mm.) of O <sub>2</sub> consumed, or CO <sub>2</sub> produced, by entire sample for entire period								Exp. No. and % O <sub>2</sub> at end	Seeds at later stage of germination (75 g.,* 16.5 hrs.)							
	Seeds at early stage of germination (150 g.,* 17 hrs.)									Seeds at later stage of germination (75 g.,* 16.5 hrs.)							
	O <sub>2</sub> consumed		CO <sub>2</sub> produced							O <sub>2</sub> consumed		CO <sub>2</sub> produced					
	O <sub>2</sub> replen- ished by O <sub>2</sub> N <sub>2</sub>		O <sub>2</sub> replen- ished by O <sub>2</sub> N <sub>2</sub>							O <sub>2</sub> replen- ished by O <sub>2</sub> N <sub>2</sub>		O <sub>2</sub> replen- ished by O <sub>2</sub> N <sub>2</sub>					
1 (13.5%)	334	330	279	302	1 (12.9%)	399	393	353	359	1 (12.9%)	399	393	353	359			
	358	353	321	320		400	393	355	350		400	393	355	350			
	400	366	347	330		431	394	373	355		431	394	373	355			
	373	347	328	321		404	377	358	350		404	377	358	350			
	1465	1396	1275	1273		1634	1557	1439	1414		1634	1557	1439	1414			
2 (14.4%)	345	323	295	282	2 (12.6%)	403	390	358	351	2 (12.6%)	403	390	358	351			
	340	302	281	274		416	400	372	365		416	400	372	365			
	326	303	281	274		441	417	388	380		441	417	388	380			
	323	275	291	274		431	395	385	370		431	395	385	370			
	1334	1203	1148	1104		1601	1602	1503	1466		1601	1602	1503	1466			

Variances obtained				Variances obtained			
D.F.		O <sub>2</sub> cons.	CO <sub>2</sub> prod.	D.F.		O <sub>2</sub> cons.	CO <sub>2</sub> prod.
Treatments	1	2500	132	Treatments	1	1722	240
Experiments	1	6561	5476	Experiments	1	650	841
T×E	1	241	110	T×E	1	10	9
Replicates	12	372	257	Replicates	12	178	109
t = 2.59				t = 3.11			
Prob. = 0.025				Prob. = < .01			

\* Weight of air dry seeds previous to soaking.

#### GERMINATING WHEAT SEEDS

The "reversal" or "switch-back" procedure was not used with germinating wheat seeds for the reason that there is a rapid change in the morphology of the seeds and in their respiration rate as germination proceeds. The second stage would be so different from the first that combining the

two sets of data would furnish results of doubtful meaning. Besides, it seemed better to deal with at least two stages separately, since the article by Reuhl (16) indicated a quite different response to  $O_2$  depletion by the seeds of even the same species in two different stages of germination.

As shown by the heading of Table VI two stages of germination were employed in these tests: (a) An early stage, consisting of the period of 17 hours following a preliminary period of 22 hours soaking in water at  $20^\circ$  C.; at the end of this period the plumules and primary roots had just emerged. (b) A later stage consisting of the period of 16.5 hours following a preliminary soaking period of 22 hours at  $20^\circ$  C., and an intermediate period of 24 hours under germinative conditions (moist atmosphere) at  $20^\circ$  C. At the end of this experimental period the plumules had elongated, the secondary roots had emerged, and roots had grown to a length of as much as 12 mm.

There were four lots of seeds with which replenishment of the  $O_2$  was made continuously with  $O_2$ , and four lots in which the  $O_2$  used up was replaced with  $N_2$ , resulting in a progressive depletion of the  $O_2$  in such containers.

After having carried out one experiment in this way a second test proceeded in a manner as nearly identical with it as possible, and the data from the two experiments were then combined for the analysis of variance.

The results are shown in Table VI. At the early stage of germination, when the  $O_2$  was allowed to become depleted from 20.9 per cent to 13.5 or 14.4 per cent  $O_2$ , there was a decrease in the rate of  $O_2$  consumed amounting to about 4.7 per cent below the control rate in the first experiment, and about 9.8 per cent in the second experiment. The "t" value was 2.59 with 12 D. F., the "t" required for probabilities of 0.05 and 0.01 being, respectively, 2.179 and 3.055. The data for  $CO_2$  produced are given in columns 4 and 5, Table VI, and they do not show a decrease in rate as a result of partial depletion of the  $O_2$  supply.

The results of the tests at a later stage of germination are shown in columns 6 to 10, Table VI. In these tests the  $O_2$  was depleted to 12.9 and 12.6 per cent respectively in the two tests. There was a decrease in the  $O_2$  consumption as a result of this depletion, the percentage decrease below the control value being 4.7 per cent in the first experiment and 5.3 per cent in the second. The "t" value was 3.11 corresponding to a probability less than 0.01. Again there was no effect upon the  $CO_2$  production as shown by the values in columns 9 and 10, Table VI, the "t" value being 1.49, well below the level of significance.

Since these experiments with germinating wheat seeds indicated that by the time the  $O_2$  supply had been depleted to about 12 to 14 per cent  $O_2$  there was an observable decrease in the rate of  $O_2$  consumption, it was decided to measure the progressive changes taking place up to this point, and

to note at about what percentage of  $O_2$  in the process of depletion there occurred a marked difference in the rates of the two groups, one with  $O_2$  maintained and the other with  $O_2$  progressively depleted. There were four lots in each group, each lot with wheat seedlings at the later stage, i.e. stage "b" (see second paragraph in this section). Computations were made

TABLE VII  
EXTENT OF OXYGEN DEPLETION NECESSARY TO RETARD OXYGEN  
CONSUMPTION IN RESPIRATION OF WHEAT SEEDLINGS

Exp. No.	Interval: start to end of	Cc. O <sub>2</sub> (0°, 760) consumed by 150 g. seeds (pre-soak wt.)								% O <sub>2</sub> at end	“t” value*
		O <sub>2</sub> replenished by O <sub>2</sub> (four replicates)				O <sub>2</sub> replenished by N <sub>2</sub> (four replicates)					
1	2 hours	81	76	70	81	75	73	76	76	19.4	.45
	3 “	133	137	133	149	132	129	138	141	18.2	.68
	4 “	185	186	181	189	182	185	183	182	17.3	.51
	5 “	241	245	239	243	238	249	237	234	16.2	.57
	6 “	293	305	301	312	291	307	300	299	14.9	.80
	7 “	355	367	362	374	349	365	360	357	13.9	1.53
	8 “	420	431	425	436	408	420	416	411	12.7	3.24
	9 “	483	496	491	499	460	476	469	466	11.7	5.56
	10 “	546	565	560	569	522	535	531	526	10.4	7.16
2	2 “	59	65	67	56	64	65	59	50	10.7	.34
	3 “	106	111	115	105	111	110	107	98	18.6	.41
	4 “	159	167	174	159	164	166	157	150	17.5	.82
	5 “	216	222	230	215	219	222	211	206	16.4	.94
	6 “	267	276	286	269	271	274	265	255	15.6	1.24
	7 “	324	334	346	327	325	327	320	311	14.3	1.80
	8 “	382	396	407	388	380	382	376	365	13.0	2.62
	9 “	435	451	470	445	429	433	425	415	11.9	3.71
	10 “	493	511	528	506	483	487	480	470	10.8	4.42
	11 “	547	572	594	564	532	534	529	518	9.8	6.15
3	2 “	67	57	55	50	65	56	50	49	10.7	.50
	3 “	116	110	113	107	113	109	109	105	18.6	.55
	4 “	170	164	168	164	165	163	165	158	17.5	.82
	5 “	221	220	224	219	214	215	218	208	16.5	1.61
	6 “	276	273	283	272	267	264	272	257	15.3	2.43
	7 “	332	333	341	331	316	320	327	312	14.2	3.44
	8 “	392	390	404	393	372	376	385	365	12.9	4.40
	9 “	445	450	459	450	420	423	437	414	11.9	6.32
	10 “	513	517	529	518	482	483	495	472	10.7	8.04
	11 “	573	578	590	582	533	530	546	525	9.6	10.48

\* "t" value required for Prob. less than .05 = 2.447; less than .01 = 3.707.

of the amount of  $O_2$  consumed at the end of hourly intervals beginning with the end of the second hour after the start, at which time convenient readings could be made on the calibrated side-tubes.

Three of such experiments were carried out in succession with the same amounts of seeds (150 g. air dry) soaked for 24 hours, then stored in air under germinative conditions for an additional 24 hours, after which they

were placed in the desiccators for the start of the test. The experiments were stopped at the end of 10 to 11 hours. The results are shown in Table VII.

Column 11, Table VII, shows the O<sub>2</sub> concentration, at the end of each interval, in the desiccators containing the lots subjected to O<sub>2</sub> depletion because of replenishment of O<sub>2</sub> consumed by adding N<sub>2</sub>. The O<sub>2</sub> concentration in the desiccators in which the O<sub>2</sub> used up was replaced with O<sub>2</sub> continuously remained at approximately the starting concentration, i.e. that

TABLE VIII  
EFFECT OF PARTIAL DEPLETION OF OXYGEN UPON THE CO<sub>2</sub>  
PRODUCTION OF WHEAT SEEDLINGS

Exp. No.	Duration, hrs.	Final O <sub>2</sub> % in the depleted lots	Cc. CO <sub>2</sub> produced when O <sub>2</sub> consumed was replenished by:	
			O <sub>2</sub>	N <sub>2</sub>
1	10	10.4	507	504
			524	499
			514	509
			519	404
3	11	9.6	496	481
			508	491
			519	506
			510	485

Total CO<sub>2</sub> produced by lots with O<sub>2</sub> maintained = 4097 cc.

Total CO<sub>2</sub> produced by lots with O<sub>2</sub> depleted = 3979 cc.

Decrease = 118 cc. = 2.9% below the control rate

Analysis of Variance of Table VIII

	D.F.	Variance	
Treatments	1	870	$F = 12.45$
Experiments	1	441	$t = 3.524$
$T \times E$	1	31	Prob. = < .01
Replicates	12	69.4	

of normal air. The entries in Table VII are the volumes of O<sub>2</sub> consumed by each of the eight separate lots from the start to the end of the time periods listed in column 2. Each of the two contrasting groups were represented by four replicates, columns 3 to 6, 7 to 10, and from these values at each period a "t" value was computed and entered in column 12. Each line in Table VII furnished one degree of freedom for treatment, and six D. F. for error (replicates within treatments). There were 54 D. F. available for computing the replicate error in Exp. No. 1, and 60 each in Exp. 2 and Exp. 3. The average value for the replicate error in each experiment was used in estimating the "t" value for each separate line. Computation of the "t"



value was made according to the method of Tippet (17, p. 81). It is seen by examining the "t" values in column 12 that these began to increase noticeably at about the 7th hour, when the O<sub>2</sub> concentration had been depleted to 13.9 per cent in the first experiment, at about the 6th hour with 15.6 per cent O<sub>2</sub> in the second experiment and at about the 5th hour with 16.5 per cent O<sub>2</sub> in the third. The changes in the "t" values are, of course, progressive and not sharp at these stages. There was a rapid increase in "t" values in the later hours, especially after the O<sub>2</sub> concentration of the depleted lots reached about 13 per cent. If we set a "t" value greater than 2.447 to represent the place at which it is probable that the depletion of O<sub>2</sub> supply is definitely retarding the rate of O<sub>2</sub> consumption, we can compute the per cent reduction in respiration rate which was reached at that stage. It is found to be 3.3 per cent in the first experiment, 4.3 per cent in the second experiment, and 4.5 per cent in the third.

It was not possible to obtain the values for the CO<sub>2</sub> produced at the end of each of the intervals for the experiments with the wheat seedlings, since removing the alkali from the desiccators would have prevented maintaining the conditions for gradually depleting the O<sub>2</sub> concentrations. However, alkali solutions were available at the end of the experiments, and from these the CO<sub>2</sub> production for the whole period of the test could be computed. Such results were obtained for Exp. Nos. 1 and 3, data for Exp. No. 2 being lost by an error in decanting the alkaline liquids. The data for CO<sub>2</sub> produced in Exp. Nos. 1 and 3 are shown in Table VIII, the results being so similar in the two experiments that combining the two sets of measurements seemed justifiable. It is seen that depleting the O<sub>2</sub> to about 10 per cent caused a reduction in the amount of CO<sub>2</sub> produced by about 2.9 per cent. Even though this effect of the oxygen depletion was small, the difference reaches the one per cent level of significance as shown by the analysis of variance.

#### DISCUSSION

These results tend to support a position somewhere between that taken by those who feel that the oxygen concentration can be varied over a wide range without having any effect upon the rate of respiration, and that taken by those who feel that any change in the oxygen concentration will have an immediate effect. With the tissues used in these tests (with the exception of pelargonium leaves, which did not furnish a conclusive test) it has not been hard to find an effect upon respiration rate, especially upon the rate of oxygen utilization, by reducing the O<sub>2</sub> concentration. The effect produced, however, has been comparatively small, a reduction in rate by about 5 per cent being caused by depleting the O<sub>2</sub> concentration of the surrounding air from the normal air content to about 13 to 15 per cent.

Experimenters who have been measuring respiration rate by methods which involved a continually decreasing concentration of oxygen in the

containers can derive a certain amount of reassurance from these tests. Unless the  $O_2$  was depleted to quite low values, it seems unlikely that their results were in error by very much as a result of the change in  $O_2$  content.

Likewise those who have been studying the effect of added  $CO_2$  on respiration rate may also derive satisfaction from these results. If they have found decreases in the rate by the addition of  $CO_2$ , it is unlikely that the depletion in  $O_2$  content which would occur simultaneously would become a serious factor until the  $O_2$  content had been reduced severely.

When it is stated that the  $O_2$  concentration was depleted to about 13 to 15 per cent, it should be remembered that these are the values finally attained at the end of the test. The  $O_2$  concentration decreased gradually during the experimental period, and these results can not be used to establish definitely the concentration of  $O_2$  at which a retarding effect began to occur. The values in column 12 in Table VII suggest the possibility that the effect begins at about 15 to 17 per cent  $O_2$ . Experiments with constant percentages of  $O_2$  maintained over long periods with tissues having reasonably constant respiration rates over such periods are needed for establishing the critical concentration, if such occurs.

The results obtained corroborate the findings of Chevillard *et al.* (5) that reducing the  $O_2$  concentration has a greater effect upon the oxygen consumption than upon the  $CO_2$  production. However, on this point further work is needed, particularly as to whether this effect is true for a wide range of  $O_2$  concentrations or whether this differential effect on the two processes takes place only when definite  $O_2$  concentrations have been reached.

There may be some objection to the use of the switch-back, or reversal, type of experiment on the basis that there may be a carry-over effect into the second and succeeding periods from the previous condition in each case. The effect of a previous sojourn in low oxygen concentrations on the respiration rate when such tissues were transferred to air containing the normal  $O_2$  content was investigated by Chevillard *et al.* (6). They found no effect if the  $O_2$  content in the previous period was 5 per cent  $O_2$  or greater. Also, Leach and Dent (9) found no extensive after-effect when they transferred seedlings to air after a previous sojourn in nitrogen; the rate rose "to the normal 'air-line' value appropriate to each particular seedling." In the present experiments the  $O_2$  concentrations did not fall below about 10 per cent in any experiment except one. The potato test described in Table II may have been in the zone in which one might expect a possible after-effect of the previous condition. In the other experiments, however, the  $O_2$  concentration was high enough at all times to justify the belief that the results were not seriously influenced in any succeeding period by the conditions prevailing previously. The rather small effects which were found also argue against the likelihood that the tissue condition was sufficiently influenced to induce a carry-over action.

If the switch-back method could not be properly used it would be necessary to use larger numbers of replicates, or to obtain a better distribution of samples for the different comparisons. This is not to say that the replicates have not shown satisfactory uniformity. The replicate error has furnished coefficients of variation (standard deviation of a single determination expressed as a per cent of the mean) about as follows: For potato tubers and wheat seedlings, 3 to 5 per cent; for geranium leaves, 4 to 6 per cent; for Jerusalem artichoke tubers, 7 to 9 per cent (the latter being high because of the necessity of using fewer tubers per sample). These may properly be regarded as low sampling errors, and yet the switch-back, or reversal, procedure in experiments in which comparisons could be made has given even smaller errors (i.e. has provided smaller probabilities for the comparisons between treatments) than was attainable by means of the replicate error (variance within treatments.)

#### SUMMARY

The oxygen consumption and carbon dioxide production of tubers of potato and Jerusalem artichoke, leaves of *Pelargonium*, and seedlings of wheat in two different early stages of germination, were measured at a constant temperature of 20° C. under two different conditions of O<sub>2</sub> supply in a closed container: (a) when oxygen was supplied to the tissues as fast as it was consumed, whereby the O<sub>2</sub> concentration was maintained at normal air content (approximately 20.9 per cent); (b) when the O<sub>2</sub> used up was replenished continually with N<sub>2</sub>, in which case the O<sub>2</sub> supply was progressively depleted to different values below normal air content, such as to 15, 12, 10 per cent, and in one case to less than 3 per cent.

There were four samples for each condition of oxygen supply in each experiment, and in order to reduce experimental error, in all cases except with wheat seedlings, use was made of the switch-back or reversal technique. Each experiment furnished its own experimental error term, whereby a test of the significance of differences could be applied.

With potato tubers, depleting the O<sub>2</sub> concentration from 20.9 per cent to 13.3 per cent over a period of 25 hours caused a reduction in O<sub>2</sub> consumption to a value 4.2 per cent below that of the control with the normal air content; under these conditions, however, there was no decrease in the rate of CO<sub>2</sub> production. When the O<sub>2</sub> was depleted to 2.4 per cent O<sub>2</sub> in 40.5 hours the O<sub>2</sub> consumption was depressed by 13.8 per cent, and the CO<sub>2</sub> production by 1.73 per cent, the latter being a small difference, it is true, but one which was significant on the basis of the statistical test applied.

With Jerusalem artichoke tubers, allowing the depletion of the O<sub>2</sub> down to a final value of 14.8 per cent in 24 hours caused a decrease of 5.4 per cent in the O<sub>2</sub> consumption but had no effect upon the CO<sub>2</sub> production. When

the depletion was made to 11.8 per cent  $O_2$  in 16.5 hours, the  $O_2$  consumption was reduced by 5.6 per cent, and the  $CO_2$  production by 1.55 per cent (again significant in spite of the smallness of the difference observed).

With *Pelargonium* leaves depletion of the  $O_2$  content to 16 per cent in 29.5 hours did not significantly affect either the  $O_2$  consumption or  $CO_2$  production.

With wheat seedlings, depletion of  $O_2$  to the range 12.6 to 14.4 per cent  $O_2$  decreased the  $O_2$  consumption by about 5 per cent, with no significant effect upon the  $CO_2$  production. However, when the  $O_2$  content was carried down to 9.6 or 10.4 per cent, the  $CO_2$  production was depressed by about 2.9 per cent of the control rate, a difference which proved to be significant according to the statistical test. When the respiration rates were measured at hourly intervals beginning with the 2nd hour and continuing to the 10th or 11th hour, it was found that a significant difference in the  $O_2$  consumption between the lots with  $O_2$  continuously maintained and the lots with  $O_2$  continually depleted occurred at about the 6th to 8th hour when the  $O_2$  content of the depleted lots had been lowered to about 13 to 15 per cent, at which time the lots with depleted  $O_2$  showed values for  $O_2$  consumption about 3 to 5 per cent below those for the controls with  $O_2$  continuously maintained.

The results with these tissues, therefore, support a position somewhere between that taken by those who believe that the  $O_2$  concentration can be reduced to low value without affecting the respiration rate, and that taken by those who believe that any reduction in the  $O_2$  content below that of normal air causes a reduction in the rate. In these experiments reductions in rate were obtained with all of the tissues except *Pelargonium* leaves, and at *terminal*  $O_2$  concentrations in the range from about 10 to 15 per cent (not regarded as excessively low). However, the extent of the reduction in rate has not been large, usually only about 4 to 5 per cent for  $O_2$  consumption, and about 2 to 3 per cent for  $CO_2$  production in the cases in which any significant effect upon  $CO_2$  production was obtained.

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# UTILIZATION OF DL-METHIONINE AS A SOURCE OF SULPHUR BY GROWING PLANTS<sup>1</sup>

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Higher plants presumably obtain their chief supply of the sulphur necessary for growth from sulphate ions absorbed by the roots. From this sulphate sulphur plants are able to form all the various sulphur compounds required for normal development. It is also presumed that sulphur present in proteins and other organic compounds in the reduced form can again be oxidized to sulphate. Evidence as to the ability of plants to oxidize various sulphur compounds to sulphate is largely lacking as to specific compounds. That plants probably can oxidize the sulphur of cystine to sulphate is indicated by the work of Mothes (4) and of Wood and Barrien (7). The results of Mothes (4) also suggest that thioglucose infiltrated into leaves may bring about an increase in sulphate sulphur.

The experiments reported in the present paper show that DL-methionine ( $\text{CH}_3\text{SCH}_2\text{CH}_2\text{CHNH}_2\text{COOH}$ ) applied in nutrient solutions is readily absorbed by growing plants and can serve as a source of sulphur for sulphur deficient plants. Under such conditions the application of DL-methionine produces increased growth and an increase in the sulphate content of the plants. Also with less severe or no sulphur deficiency the application of DL-methionine results in an increased sulphate content.

These experiments offer strong evidence, therefore, that the plants studied (tomato, *Lycopersicon esculentum* Mill., and tobacco, *Nicotiana tabacum* L. var. Turkish) can oxidize the sulphur of DL-methionine to sulphate. Unequivocal proof is perhaps lacking since possible oxidation in the cultures before absorption is not entirely excluded. In view of the fact that large amounts of added DL-methionine are shown to be absorbed unchanged and in view of the uniformity of the responses under the various conditions employed, it seems logical to conclude, in the absence of evidence to the contrary, that the effects produced by the addition of DL-methionine to the nutrient media are the result of the ability of the growing plants to utilize the absorbed DL-methionine as a sulphur source.

## RESULTS

### EXPERIMENTS WITH TOMATO PLANTS

#### *First Tomato Series*

For this test tomato seedlings of the Bonny Best variety were transferred to sand cultures in 5-inch clay pots and supplied with nutrients by

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the drip culture method as described by Shive and Robbins (6). The seedlings had only two true leaves when transferred to the sand. The complete nutrient solution applied was made up according to Formula I of Shive and Robbins (6) and contained the following salts in the molar concentrations indicated:  $\text{KH}_2\text{PO}_4$ , .0023;  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , .0045;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , .0023;  $(\text{NH}_4)_2\text{SO}_4$ , .0007. In addition a minor element solution was added so that the final solution contained 3 p.p.m. of iron as ferric tartrate, 0.50 p.p.m. of manganese as  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 0.25 p.p.m. of boron as  $\text{H}_3\text{BO}_3$ , and 0.125 p.p.m. of zinc as  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ . For the minus sulphur solution the corresponding chlorides were substituted in equivalent amount in each case in which sulphates are used in the complete solution including the minor element solution. One liter of nutrient was supplied each culture daily five times weekly. For the other two days distilled water was added to the cultures as needed.

In this series the treatments included a plus sulphate solution, a minus sulphate solution, a minus sulphate solution plus 30 mg. of DL-methionine per liter and a minus sulphate solution plus 8 p.p.m. of  $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ . The use of a solution containing barium was connected with other experiments involving the addition of barium to nutrient media as a means of reducing available sulphur in sulphur nutrition experiments. These experiments with barium are not reported in this paper. However, since techniques including the use of barium are a part of two of the succeeding series concerned with DL-methionine utilization, some of the results of these unpublished investigations will be mentioned in later sections of this paper.

The cultures of these four treatments were set up in the greenhouse (December 12, 1946) in rows of three with the same treatment in each row and the rows replicated three times. The relative order of the treatments was determined by lot. Appraisal of the relative growth by the method of ranking (2) indicated early in the experiment (after about 20 days) the superiority of the plus sulphate and the DL-methionine cultures over the other two. Availability of the sulphur from DL-methionine was thus suggested early by this growth response. A photograph taken 46 days after the start of the experiment is shown in Figure 1. It is evident that the growth with DL-methionine is much better than in the minus sulphate solution.

One day after the photograph was taken, or 47 days after the start of the experiment, the plants were sampled. Individual fresh weights of the tops in each culture were obtained and it was therefore possible to evaluate the growth response statistically. An analysis of variance showed that a difference between the total weight of two treatments of 89 is required for 19:1 significance and of 120 for 99:1 significance. Examination of the data in Table I shows that the increased growth in the plus sulphate and DL-methionine lots over that of the minus sulphate and minus sulphate



FIGURE 1. Typical plants from drip culture experiment with tomato on the utilization of DL-methionine as the sole source of added sulphur. Left to right, plus sulphate solution, minus sulphate solution plus 30 mg. DL-methionine per liter, minus sulphate, and minus sulphate plus 8 p.p.m.  $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ .

plus barium lots is highly significant. The somewhat lesser growth in the DL-methionine series as compared to the plus sulphate is not significant at odds of 19:1.

Analyses for sulphate were made on the expressed juice. This was obtained by grinding the plants through a food chopper using a fine cutter and squeezing out the juice by hand through cheesecloth. The juice was then heated to  $80^\circ \text{C}$ . and filtered after cooling. All values for sulphate reported in this paper are based on analyses made on such heated and filtered juice.

TABLE I  
RESPONSE OF TOMATO PLANTS IN SAND CULTURE TO THE PRESENCE  
OF DL-METHIONINE IN THE NUTRIENT MEDIUM

Treatment	Fresh weight of tops, g.	$\text{SO}_4$ , mg. per 100 cc. expressed juice
Plus sulphate	476**	221
Minus sulphate plus DL-methionine	409**	15
Minus sulphate	203	< 2
Minus sulphate plus 8 p.p.m. $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$	177	< 2

\*\* Increased fresh weight of these treatments over the other two highly significant statistically.



Values for sulphate are expressed as milligrams  $\text{SO}_4$  per 100 cc. expressed juice. Volumes of juice taken and other conditions of the sulphate determinations were such that contents above 1 to 2 mg. per 100 cc. could be determined. It is seen from Table I that not only did the use of DL-methionine give increased growth over the minus sulphate controls approximately equal to that of the cultures receiving adequate sulphate but the DL-methionine plants also contained additional sulphate. In other words the added DL-methionine furnished enough sulphur to about double the fresh weight of tops produced and in addition to give an appreciable sulphate content in the expressed juice. Expressed juice was also obtained from the roots. No precipitate for sulphate was noted in any of the treatments except the plus sulphate, the roots of which contained 52 mg.  $\text{SO}_4$  per 100 cc. of juice.

The expressed juices were examined for the presence of methionine by the method of McCarthy and Sullivan (3). The top juice from the DL-methionine series was found to contain about 1 mg. methionine per 5 cc. (some interference from natural color present in the juice) and the root juice 2.5 mg. in 5 cc. A positive test was not obtained from the plus sulphate plants, either tops or roots. The presence of these relatively large amounts of methionine shows that much of the DL-methionine, at least, added to the medium has been absorbed unchanged. Presumably then the beneficial effects have been the result of utilization of the absorbed DL-methionine by the growing plants.

### *Second Tomato Series*

In view of the results of the experiments described above a second series of tomato cultures to supplement the information obtained in regard to the utilization of DL-methionine was carried out. Seedlings with only two true leaves, again of the Bonny Best variety, were transferred to sand cultures on February 4, 1947. Cultures were arranged in rows of three with the same treatments in the row and the order of the treatments determined by chance. The treatments were replicated twice. The following solutions were used: plus sulphate, minus sulphate, minus sulphate plus 30 mg. DL-methionine per liter, minus sulphate plus 24 mg. L-cystine per liter, minus sulphate plus 24 mg. L-cystine and 30 mg. DL-methionine per liter, minus sulphate plus 60 mg. DL-methionine per liter and minus sulphate plus 29 mg. sodium sulphate per liter. Quantities are adjusted to give equal sulphur content except for the doubled DL-methionine treatment and for the treatment with both L-cystine and DL-methionine.

Typical plants from each series photographed 32 days after the start of the test are shown in Figure 2. The superiority of all other treatments over the minus sulphate lot is evident. The following day the plants were sampled and data for the fresh weight of tops and for the sulphate content of

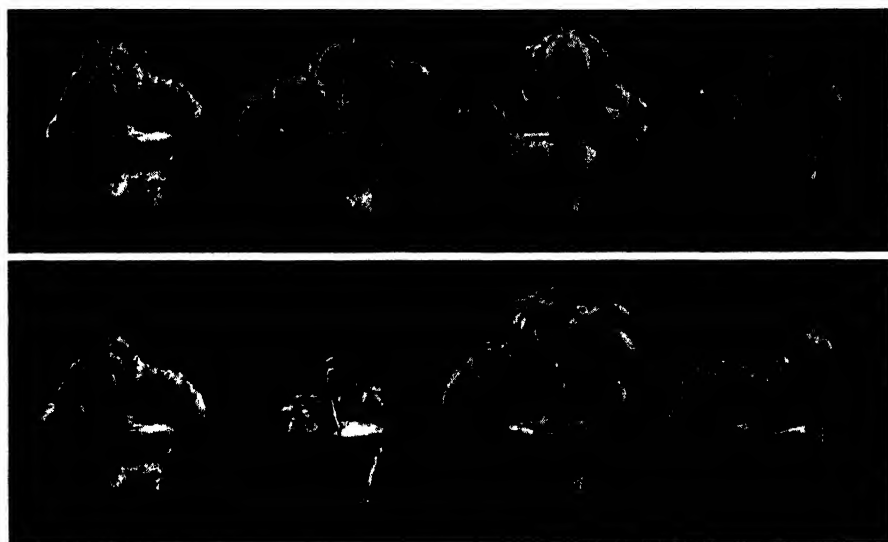


FIGURE 2. Tomato plants from DL-methionine, L-cystine series. Treatments, left to right, upper row, plus sulphate, minus sulphate plus 30 mg. DL-methionine per liter, minus sulphate plus 25 mg. L-cystine per liter, minus sulphate plus 25 mg. L-cystine and 30 mg. DL-methionine; lower row, plus sulphate, minus sulphate, minus sulphate plus 29 mg.  $\text{Na}_2\text{SO}_4$  per liter, minus sulphate plus 60 mg. DL-methionine per liter.

the expressed juices are given in Table II. An analysis of variance conducted on the logarithms of the fresh weights showed the decreased yield

TABLE II  
EFFECT OF PRESENCE OF DL-METHIONINE AND L-CYSTINE IN NUTRIENT MEDIUM ON THE GROWTH AND SULPHATE CONTENT OF TOMATO PLANTS

Treatment	Fresh weight of tops, g.	$\text{SO}_4$ , mg. per 100 cc. expressed juice
Plus sulphate	139	193
Minus sulphate	76**	4
Minus sulphate plus 30 mg. DL-methionine	141	15
Minus sulphate plus 24 mg. L-cystine	177	32
Minus sulphate plus 24 mg. L-cystine and 30 mg. DL-methionine	150	29
Minus sulphate plus 60 mg. DL-methionine	118	30
Minus sulphate plus 29 mg. $\text{Na}_2\text{SO}_4$	179	46

\*\* Difference in yield between this lot and other treatments highly significant.

of the minus sulphate lot as compared to all other treatments to be highly significant statistically but differences among the other treatments are not significant. Both L-cystine and DL-methionine produced an increase in the sulphate content of top juice. Examination of the root juices for sulphate showed a very low content for all except the plus sulphate, the root juice of which contained 57 mg.  $\text{SO}_4$  per 100 cc.

Approximate methionine content of the expressed juice of the root samples, expressed as milligrams in 5 cc. was found to be as follows: minus  $\text{SO}_4$  plus 60 mg. DL-methionine, 5; minus  $\text{SO}_4$  plus 30 mg. DL-methionine and 24 mg. cystine, 3; minus sulphate plus 30 mg. DL-methionine, 1.5; no test in plus sulphate or minus sulphate plus L-cystine samples. Positive tests were also obtained for the juice from the tops of the minus sulphate plus 30 mg. DL-methionine, minus sulphate plus 60 mg. DL-methionine, and minus sulphate plus DL-methionine and L-cystine lots but the quantities were difficult to evaluate because of the interfering color of the juices. The top juice from the plus sulphate plants did not give a positive test. These results thus again show that large quantities of the added DL-methionine are absorbed unchanged. It would thus appear reasonable to conclude that the increased sulphate contents obtained are the result of oxidation of DL-methionine within the plants rather than the result of the action of organisms or other factors on the DL-methionine in the cultures before absorption.

#### *Further Experiments on the Oxidation of DL-Methionine to Sulphate in Tomato Plants*

For this experiment tomato plants were used which had been grown in sand in 5-inch pots supplied with saucers. The nutrients were added to the cultures three times weekly and in adding additional water as needed care was taken not to splash solution and therefore nutrients out of the saucers. The nutrient solution used had the same composition as that used for drip cultures but it was applied at twice the drip culture concentration. Each culture received 150 cc. at each application. Seedlings which had previously been started in sand with a minus sulphate solution were transferred to the cultures after two true leaves had formed. Forty plants received the minus sulphate solution and 10 the complete solution. Necessary supplementary additions of water were made with distilled water. In spite of the use of distilled water and the exclusion of sulphur from the nutrient solution the minus sulphate plants accumulated considerable sulphate as shown by an analysis of juice from a small sample of leaves collected 27 days after the start of the cultures (minus sulphate, 95 mg.  $\text{SO}_4$  per 100 cc., plus sulphate, 235 mg.).

In order to reduce the amount of sulphate in these plants for the tests with DL-methionine barium was added. This use of barium is based on

fairly extensive unpublished experiments. These tests have shown that controlled additions of barium will readily reduce the sulphate content of the expressed juices but do not have much effect on growth unless barium ions are present in excess in the expressed juices. Under such conditions growth may stop entirely but the plants stay alive for long periods and growth will again be resumed if sulphate is added. In the present series, then, starting with a concentration of sulphate of 95 mg.  $\text{SO}_4$  per 100 cc. juice, gradual additions of barium were made until practically all of the  $\text{SO}_4$  was eliminated from the expressed juice from the leaves.

Four days after the analysis reported above and 31 days after the start of the series 140 mg. of  $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$  was added to each of 30 of the minus sulphate plants. Two days later analyses of a small sample of leaf material (one leaf near the tip from each of five plants) gave the following results for sulphate, expressed as mg.  $\text{SO}_4$  per 100 cc. juice: minus sulphate plus barium, 83; minus sulphate, 102; plus sulphate, 317. Within the next week two more additions of barium, 280 mg.  $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$  to each culture, were made. One day after the last addition the 30 pots were changed to drip cultures. They were divided into two lots, one receiving the regular minus sulphate drip culture solution used in the earlier series plus 5 p.p.m. of  $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$  and the other receiving the same solution, including the barium, plus 30 mg. of DL-methionine per liter. An analysis made one day after the start of the drip cultures gave a content of 17 mg.  $\text{SO}_4$  per 100 cc. of leaf juice. Leaves from the DL-methionine series were mixed with the others since DL-methionine had been added for only one day. In order to reduce the sulphate content still further the barium addition was increased to 15 p.p.m. Four days after the quantity of barium was thus increased and six days after DL-methionine was first added analysis of a small sample showed the sulphate content to be 40 mg. for the DL-methionine lot and 27 for the minus sulphate lot. It is seen that the effect of the DL-methionine in increasing the sulphate content has already become evident. After six more days the increased barium additions resulted in reducing the sulphate content of both series so that now the lot without DL-methionine gave no precipitate for sulphate while the DL-methionine treated lot contained 16 mg. per 100 cc. expressed juice. In order to avoid an excess of barium with resultant barium toxicity the barium addition was again reduced to 5 p.p.m. of  $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$  and maintained at this level for the remainder of the experiment.

Another analysis was made two days after the one reported above and again the expressed juice from the minus sulphate lot without DL-methionine gave no sulphate precipitate. Expressed juice from the leaves of the methionine series contained 11 mg. per 100 cc. Examination of the juices for methionine by the method of McCarthy and Sullivan (3) indicated the presence of about 1 mg. in 5 cc. in the DL-methionine lot. The

estimate was complicated by the natural color of the juice. An analysis for sulphate made 6 days later gave values of 42 and 6 for the DL-methionine and no DL-methionine leaves respectively. Examination at this time of fruit from both series failed to yield a precipitate for sulphate or for barium in either lot.

The above analyses were made on small portions of the material, usually 5 to 10 leaves per sample, the leaves being picked, one leaf per plant, near the tip. Twenty-two days after the start of the addition of DL-methionine and two days after the last analysis reported above the entire plants were sampled. Juice was expressed separately from the tips and from leaf samples according to position along the stem as indicated in Table III. In accordance with the preliminary samples the sulphate content of the DL-methionine lot was much higher (usually twice as high or more)

TABLE III  
INCREASE IN SULPHATE CONTENT FOLLOWING ADDITION OF  
DL-METHIONINE TO GROWING TOMATO PLANTS

Plant part	Fresh weight, g.		SO <sub>4</sub> , mg. per 100 cc. expressed juice	
	DL-Methionine added	No DL-methionine	DL-Methionine added	No DL-methionine
Tip	69	59	21	< 2
Leaf at tip	104	73	29	8
Second leaf	156	136	30	11
Third leaf	224	194	40	12
Fourth leaf*	297	267	35	22
Roots	205	190	29	10

\* Remaining leaves, amounting to 1510 grams in the DL-methionine lot and 1378 grams in the lot without DL-methionine, were not sampled.

than that of the controls. Leaves farther down than the fourth from the tip were not sampled for sulphate content.

It will be noted that the weights are somewhat higher in the case of the DL-methionine plants than in those without DL-methionine. This experiment was not designed for a test on growth effects so that it is not possible to state whether the weight differences noted are significant. If, of course, at any time sulphur was limiting for growth in this series, then in line with the results found in the earlier tomato series DL-methionine would be expected to make up the deficiency. In addition to the leaf and root weights shown in Table III, the fresh weights of fruit and stems of the two series were as follows: fruit, DL-methionine, 997 g. (plus 337 g. sampled 2 days earlier); no DL-methionine, 583 g. (plus 363 g. sampled earlier); stems, DL-methionine, 1257; no DL-methionine, 1326.

The expressed juice of the roots was found to contain about 2.5 mg. of

methionine in 5 cc. in the DL-methionine series while no test was obtained with the roots from the lot without added DL-methionine.

It will be recalled that the 30 plants used in these tests were a part of a larger series in static sand cultures. Analyses of leaves from minus sulphate and plus sulphate plants at the start of the DL-methionine experiment are given earlier. It may be of interest to report the sulphate content of leaves from the plants which were continued in the static cultures while the 30 plants were used in the drip culture DL-methionine series. Analyses of leaves made at the time the DL-methionine series was sampled gave the following results in mg. per 100 cc. expressed juice: plus sulphate, 650; minus sulphate, 209; minus sulphate with 1 g.  $\text{BaCO}_3$  mixed in sand one month before sampling, 150.

The results obtained with this series, showing marked increase in sulphate content on addition of DL-methionine, thus confirm the results of the first two series described.

#### *DL-Methionine as a Corrective for Barium Toxicity*

Unpublished experiments with the use of barium in nutrient solutions have shown that when enough barium is added to yield an appreciable quantity of barium ions in the expressed juices growth is seriously retarded or may cease entirely. Under these conditions the addition of sulphate will bring about resumption of growth. In some of these experiments the sulphate was added through an auxiliary root system developed on a portion of the stem while the main root system was kept undisturbed under high barium conditions. With this technique it is possible to have two plants with differing treatments in the same pot and thus in the same environment as far as the main root systems are concerned.

The method used in supplying the corrective chemical can be seen by an examination of Figure 3. It is most clearly shown in the plant on the extreme left. A short distance above the sand level about three or four inches of the stem has been split in two with a sharp, thin, steel blade and half of the stem severed horizontally. The portion of stem thus available has been immersed in a solution in a 50 cc. beaker in which a considerable root system has developed. In the illustration a 500 cc. Erlenmeyer flask is connected with the beaker in such a way as to furnish a constant level of solution in the beaker. With this technique it is possible to introduce chemicals into growing plants rather more directly than through the regular root system and certain changes which might occur with chemicals added to the sand cultures may be minimized.

Since it had been found that sulphate would correct barium toxicity, compounds which the plant can oxidize to sulphate would be expected to have similar favorable effects. Accordingly DL-methionine was tested in this way, and preliminary results have indicated that DL-methionine will

counteract the deleterious effect of barium. One such test is described in detail below.

Tomato plants about 10 inches high were transferred to 10-inch pots in sand, two plants per pot. They were supplied with the nutrient solution used for static cultures in the previous series and in addition received 280 mg.  $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$  at each nutrient application (three times weekly). The plants were watered with tap water. After several weeks, examination of



FIGURE 3. Photograph showing the effect of DL-methionine in producing growth from leaf axils in tomato plants (with tips removed) exhibiting barium toxicity. Plants of equal height when DL-methionine addition through split stem was started. Plant with added DL-methionine on left in each case.

some of the material for barium with potassium rhodizonate (5) indicated no free barium and the amount of barium added was therefore increased 50 per cent to 420 mg. per culture. Thirty-nine days after the start of the cultures, at which time the plants were about 25 inches tall, a portion of the stem was split as described above and the regular drip culture minus sulphate nutrient solution used to induce root development prior to DL-methionine addition. After another week the amount of barium added was again increased, this time to 630 mg. for each application and this quantity was maintained throughout the experiment.

The split stem of all plants was kept in the minus sulphate solution for

24 days during which good root development had occurred. The plants were now from 28 to 40 inches tall. For the DL-methionine studies the plants in three pots were cut down to 22 inches and all growth from leaf axils removed. The sturdier of the two plants in each case was kept on the minus sulphate solution while the other was changed to minus sulphate plus 30 mg. DL-methionine per liter. Observations made as to the new growth appearing from the leaf axils indicated within about 10 days the superiority of the plants receiving DL-methionine. Plants in two of the pots are shown in Figure 3 as they appeared 36 days after the start of the addition of DL-methionine. In both cases the DL-methionine treated plant is shown on the left. The new growth with DL-methionine amounted to 48 g. in the pot illustrated on the left while no new growth occurred in the minus sulphate plant. In the pot shown on the right the plant treated with DL-methionine yielded 46 g. of new growth and the control 6 g. In a third pot, not shown, 27 g. of new growth were formed by the DL-methionine plant and 20 g. by the control. The quantities of the solution containing DL-methionine which had been absorbed through the split stem roots during the 36 days of treatment were approximately 2150, 1600, and 850 cc., respectively. As would be expected, more solution was absorbed the greater the growth produced. Similarly, the minus sulphate plants took up comparatively little solution. Examination of the solution in the DL-methionine beakers for sulphate after the experiment had been continuing for about 30 days yielded no precipitate, indicating no observable oxidation of DL-methionine to sulphate outside the plant.

The expressed juice obtained from the new growth did not give a precipitate for sulphate in any of the samples. However, composite samples of the old leaves which had been on the plants when the addition of DL-methionine was started gave a sulphate content of 4 mg. per 100 cc. expressed juice for the plants which had received DL-methionine and a barium content equivalent to 44 mg.  $\text{SO}_4$  per 100 cc. for the plants receiving only the minus sulphate solution.

#### FORMATION OF SULPHATE FROM ABSORBED DL-METHIONINE BY TOBACCO PLANTS

For these experiments with tobacco a sand culture series originally started by Dr. W. J. Youden for another purpose was kindly made available to the author. Tobacco plants, three to four inches tall, were transferred to sand cultures in one-gallon glazed earthenware jars with a drainage opening at the bottom. Two plants were placed in each jar. Prior to being transferred to the gallon jars the plants had been grown from seed in the absence of added sulphur. There were 50 cultures consisting of 25 receiving single strength nutrient solution and 25 receiving double strength solution. Each group of 25 was further divided into 5 lots of 5 cultures each



which received 0, 2.4, 4.8, 7.2, and 9.6 mg. sulphur as  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  dissolved in 10 cc. of water five times weekly. The basic solution added to the cultures (single strength) had the following composition expressed as moles per liter:  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , .0064;  $\text{KNO}_3$ , .0033;  $\text{KH}_2\text{PO}_4$ , .0009;  $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , .0006;  $\text{NH}_4\text{Cl}$ , .0014;  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , .0004. In addition

TABLE IV  
EFFECT OF DL-METHIONINE IN INCREASING THE SULPHATE CONTENT  
OF TOBACCO LEAVES AND ROOTS

Days after start of DL-methionine addition	Plant part	Treatment*	SO <sub>4</sub> , mg. per 100 cc. expressed juice	
			DL-Methionine added	No DL-methionine
Day before	Leaves	S 0	35	
		S 4.8	124	
		S 9.6	167	
		D 0	9	
		D 4.8	64	
		D 9.6	92	
6	Leaves	D 0	16	7
7	Leaves	S 0	66	29
		S 2.4	83	64
		D 2.4	33	29
14	Leaves	S 0	74	49
		S 2.4	77	78
		D 0	52	18
		D 2.4	40	31
21 to 30	Roots	S 0	55	27
		S 2.4	68	51
		S 4.8	88	63
		S 7.2	83	57
		S 9.6	89	62
		D 0	25	< 2
		D 2.4	43	6
		D 4.8	49	16
		D 7.2	73	27
		D 9.6	69	44

\* S=single strength nutrient solution, D=double strength. Numbers from 0 to 9.6 refer to milligrams S as  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  added 5 times weekly for 29 calendar days prior to start of addition of DL-methionine.

borax was added to give a final concentration of 0.08 p.p.m. of boron and  $\text{MnCl}_2$  to give 2 p.p.m. of manganese. Iron was added from time to time in the form of a few drops of a dilute solution of ferric chloride. Each culture received 100 cc. of the above solution, poured on the sand five times weekly. The double strength cultures received 100 cc. of a solution double in strength to that described. Distilled water was used to furnish additional water as needed.

When these cultures were adapted for the DL-methionine experiments they had received sulphate for 29 calendar days. The addition of sulphate was then stopped and each lot of five jars divided into two groups (two treated, three untreated, and three treated, two untreated, alternately) one group in each case consisting of cultures receiving 15 mg. methionine dissolved in 10 cc. of water five times weekly and the other group serving as a control.

Analytical figures showing the effect of methionine in increasing the sulphate content are given in Table IV. Marked increases are evident six to seven days (the earliest time samples were taken) after the start of the addition of the DL-methionine. Again the evidence is clear that the plants have changed sulphur of DL-methionine to sulphate unless one would assume that the increases in sulphate arise from DL-methionine oxidized in the cultures prior to absorption. Analyses for methionine in the root samples showed a content of between 1 and 2.5 mg. per 5 cc. in plants to which DL-methionine had been added. Control plants did not give a positive test.

#### DISCUSSION

That DL-methionine can be oxidized to sulphate in the animal body has been known for some time and it is believed that both the D and L forms can be utilized (1, p. 253). As far as the author is aware this is the first case in which the fate of added DL-methionine in plants has been studied. It is of interest that in the present experiments, in spite of an increase in sulphate content and in some instances considerably greater growth with consequent sulphur utilization therefor, a considerable accumulation of methionine occurs in the tissues. This might indicate inability of the plants to metabolize rapidly the relatively large quantities absorbed (many times the concentrations normally found) or, if the plants can utilize only the natural L isomer, accumulation of the unacceptable D form could account for the high concentrations encountered. Isolation of the methionine remaining in the tissues and determination of the relative quantities of the optical isomers present would show whether one form is used in preference to the other.

It would be of interest also to determine whether supplying such relatively large quantities of DL-methionine has any effect on the amount of protein methionine in the tissues.

#### SUMMARY

DL-Methionine added to nutrient solutions used in sand cultures was readily absorbed by growing tomato and tobacco plants. Such plants had an increased content of sulphate in their expressed juices. In some experiments with tomato, in which growth was limited by sulphur deficiency, the addition of DL-methionine produced increased growth as well as a higher

sulphate content. These results offer strong evidence that tomato and tobacco plants can oxidize DL-methionine to sulphate and that DL-methionine can therefore be a source of required sulphur.

In experiments with tomato plants in which growth was limited by the presence of an excess of barium ions the addition of DL-methionine through auxiliary roots developed on a portion of the stem induced growth and reduced the barium content of the expressed juice of the plants. It is believed that these results offer additional evidence that DL-methionine is oxidized to sulphate in the growing plants since additions of sulphate will similarly counteract toxicity arising from an excess of barium.

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# A COMPARISON OF THE INFECTIVITY OF DIFFERENT PREPARATIONS OF TOBACCO-MOSAIC VIRUS WITH THEIR ABILITY TO PRECIPITATE SPECIFIC SERUM ANTIBODY<sup>1</sup>

HELEN PURDY BEALE AND MARY E. LOJIKIN

## INTRODUCTION

After mild treatment with chemical reagents such as formaldehyde, nitrous acid, and dilute hydrogen peroxide, or as a result of irradiation with ultra-violet or X-rays, several of the plant viruses have been found to lose their infectivity but to retain other characteristic physico-chemical properties including the ability to combine with specific serum antibody. If the treatment is so drastic that the virus protein becomes denatured, the serologic activity is lost as well as the infectivity and other properties possessed by the native virus (1, p. 118-119, 194-210).

It seems reasonable to assume that a high-molecular-weight, complex antigen such as tobacco-mosaic-virus nucleoprotein, has a number of groups capable of combining with specific serum precipitin, so that virus which has been rendered non-infectious but has not been denatured will retain unaltered groups which will react with antibody. It is not known whether that portion of virus responsible for infectivity actually combines directly with antibody to bring about the neutralization reaction characteristic of virus-antiserum mixtures in general. However, it has been shown that the infectivity of the virus thus inactivated can be restored by various methods involving dissociation of antigen and antibody or destruction of the antibody by specific enzymic reaction leaving the virus intact (2, p. 301-302; 4, p. 951-959).

During quantitative studies on the precipitin reaction of the tobacco-mosaic virus-antiserum system (3), virus preparations purified at different times have been used. Since the infectivity of the oldest virus preparation, No. 2, was found to be less than that of the more recently purified ones, a comparison of the ability of the various preparations to combine with serum precipitin on a quantitative basis was made in order to determine whether loss of infectivity might be accompanied by an appreciable decrease in the amount of precipitate formed. Under the conditions of the experiment no correlation between these two factors was demonstrable.

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## EXPERIMENTAL PROCEDURE

## MATERIALS AND METHODS

*Purification of virus.* Tobacco-mosaic virus, preparation No. 2, was supplied by Dr. W. M. Stanley of the Rockefeller Institute for Medical Research in February, 1942, in the form of pellets obtained by the ultracentrifugation of a solution of a stock supply. Preparations Nos. 3 and 4 of the same virus were partially purified in the laboratories of Boyce Thompson Institute for Plant Research in a Sharples Super-Centrifuge from the expressed juice of macerated frozen tissue from tobacco plants, *Nicotiana tabacum* L. of a Turkish variety, grown in the greenhouse and used for the multiplication of the virus. A further purification of the two preparations was subsequently made in the ultracentrifuge in Dr. Stanley's laboratory at Princeton, N. J. Virus preparation No. 3 was made in February, 1944, and No. 4 in February of 1945. A troublesome dark pigment was present in the last preparation which was not completely removed by several runs in the ultracentrifuge. The reason for the occurrence of this contaminating substance is not clear since the conditions for the multiplication of the virus and the purification procedures for the Nos. 3 and 4 preparations were similar.

*Testing of virus preparations for possible presence of contaminating substances.* Preparation No. 2 was tested previously (3, p. 389) with antiserum to normal-tobacco-plant protein and was found to contain an amount of normal-plant protein not in excess of 1.5 per cent. Nos. 3 and 4 were subjected to electrophoretic separation made possible through the cooperation of Dr. D. H. Moore of the College of Physicians and Surgeons, Columbia University. The tests were made with a Tiselius apparatus on preparations of virus in a barbiturate buffer of about 0.1 ionic concentration at pH 8.6. The virus nucleoprotein was subjected to the shortest period of dialysis required for obtaining equilibrium since dialysis against the buffer partially inactivated the virus. In preliminary tests, no separation was obtained in a 0.02 M phosphate-0.15 M NaCl buffer at pH 7.4 and no appreciable separation in a borate buffer at pH 8.2. In the barbiturate buffer at pH 8.6, preparation No. 3 migrated as a single component but the No. 4 preparation separated into three components. An electrophoretic run was then made on the middle fraction, constituting the largest part of the preparation, and this fraction now migrated as a single component.

*Determining the infectivity of the virus preparations.* The infectivity of the various preparations of tobacco-mosaic virus was compared by determining the numbers of local necrotic lesions induced in the leaves of *Nicotiana glutinosa* L., which had been rubbed with a cheesecloth pad thoroughly moistened with virus. Either an entire leaf or only a half-leaf was inoculated according to the Latin-square (7) arrangement and the

results were subjected to an analysis of variance in order to ascertain whether the observed differences were statistically significant. Preparations Nos. 2, 3, and 4 were compared, each with the other, in this manner and the results were tabulated (Table I). In other cases, the comparison was made by inoculating opposite halves of the same leaf with different virus preparations and by using a modified Student's method of computation to determine significant differences, but since the results of the latter method entirely confirmed the findings of the former they were not included in Table I.

### RESULTS OF INFECTIVITY TESTS

#### COMPARISON OF THE INFECTIVITY OF THE VIRUS PREPARATIONS

In Table I the results obtained with equal and unequal concentrations of the same inoculum were recorded as a basis for evaluating the method employed with a view to determining later the approximate relative infectivity of the different preparations, Nos. 2, 3, and 4. When duplicates were compared, in three out of four cases, no significant difference in infectivity was obtained. In 17 tests where one inoculum was from one and one-third to twice as concentrated as the other the difference was significant in nine of the cases. Where the difference was four-fold in two cases, both showed a significant difference.

When the three different preparations were compared at approximately equal concentrations of Kjeldahl protein, in all nine cases the differences in infectivity between Nos. 2 and 3 were highly significant, the latter inducing the larger number of lesions. In the case of Nos. 2 and 4, the latter was found to be significantly more infective than No. 2 in four tests, but in the fifth instance the difference was not significant. In five of ten comparisons between Nos. 3 and 4, the former induced a significantly larger number of lesions than No. 4, while in the other five, no significant difference was shown.

The preparations were also compared at unequal concentrations. When No. 2 was inoculated at two to four times the concentration of No. 3, eight of the 13 pairs of inoculums tested showed that No. 3 was still significantly more infective, while the remaining five gave no significant difference. Not until the amount of Kjeldahl protein in No. 2 was eight times that present in the No. 3 inoculum, was the greater number of lesions induced by the former and neither of the two tests resulted in a significant difference. When Nos. 3 and 4 were compared, when the latter was inoculated in twice the concentration of the former, in two of five tests No. 4 proved significantly more infective, and in the remaining three there was no significant difference. In only one instance was No. 4 compared at a concentration as great as four times that of No. 3, but the difference in

TABLE I  
DETERMINING THE INFECTIVITY OF PURIFIED PREPARATIONS OF  
TOBACCO-MOSAIC VIRUS ON NICOTIANA GLUTINOSA L.

Date of experiment	Total No. leaves inoculated with each inoculum	Virus preparation			Total No. lesions induced	No. lesions†† indicating significant difference when	
		No.	Kjeldahl protein in			P = 5%	P = 1%
			Inoculum	Mg./cc.			
Nov. 6, 1944	18	3	A C	0.090 0.045	1430 1151	333	445
		2	E F	0.104 0.104	681 423		
Feb. 28, 1945	72†	3	A B C	0.103 0.077 0.052	3792 3263 2943	472	621
		2	D E F	0.101 0.076 0.051	2850 2179 1655		
Apr. 3, 1945	36	2	A B	0.090 0.050	1339 1518	452	596
		3	C D	0.051 0.025	2420 1817		
		4	E F	0.008 0.049	2737 2338		
May 14, 1945	36	2	A B	0.100 0.050	832 625	436	575
		3	C D	0.050 0.025	2247 1839		
		4	E F	0.049 0.025	2407 1326		
June 27, 1945	36†	2	A B	0.202 0.051	606 403	173	227
		3	C D	0.050 0.025	770 537		
		4	E F	0.048 0.024	531 436		
July 20, 1945	36†	2	A B	0.200 0.050	3082 2386	462	609
		3	C D	0.050 0.025	3228 2897		
		4	E F	0.049 0.024	3087 2485		

TABLE I—(continued)

Date of experiment	Total No. leaves inoculated with each inoculum	Virus preparation			Total No. lesions induced	No. lesions†† indicating significant difference when	
		No.	Kjeldahl protein in			P = 5%	P = 1%
			Inoculum	Mg./cc.			
Apr. 17, 1946	30†	2	A D	0.050 0.050	261 373	66	88
		3	B D	0.050 0.050	371 408	62	82
		4	D C	0.050 0.060	422 358	83	111
Oct. 11, 1946	48	3	D E	0.050 0.050	6104 6786	731	965
		4	F	0.050	5247		
Oct. 15, 1946	24-30†	3	D	0.025	848	136	180
		4	F	0.025	462		

† In this experiment, the half-leaf was used as a unit instead of the whole leaf.

†† The number of lesions required for significant differences was obtained by the analysis of variance.

infectivity was highly significant with the No. 4 inoculum proving the more infective. Nos. 2 and 4 were also compared when No. 2 was two to four times as concentrated as No. 4, and one-half of the eight tests resulted in a significant difference with the latter possessing the greater infectivity, while the remaining four cases showed no significant difference. When the concentration of the No. 2 inoculum was increased to eight times that of No. 4, both tests showed No. 2 caused the larger number of lesions, resulting in one case that was significant. From this consideration of the results, it is evident that No. 3 is the most infective virus preparation. No. 4 is not so infective and No. 2 is much less infective than either Nos. 3 or 4. From the results the relative infectivity of No. 2 can be regarded as approximately one-seventh of that of No. 3. No correction has been made for the presence of non-virus protein known to be present in preparations Nos. 2 and 4 for reasons which will be considered in the discussion.

#### QUANTITATIVE SERUM-PRECIPITIN REACTION

##### DETERMINING THE ABILITY OF THE VIRUS PREPARATIONS TO PRECIPITATE SPECIFIC SERUM ANTIBODY

*Preparation of antiserum to tobacco-mosaic virus.* Horse antiserum was prepared by Dr. Lester Reddin, Jr., through the courtesy of Lederle Labo-



ratories, Pearl River, N. Y. A horse was co-immunized with swine erysipelas antigen and tobacco-mosaic nucleoprotein injected separately, which it was agreed should not interfere with the development of antibody specific to the plant virus. Preparation No. 3, which as previously stated migrated as a single component when subjected to the electrophoretic test, thus fulfilling the requirements for purity, was injected subcutaneously in approximately 43 mg. doses. Eight injections were given at intervals varying from four to six days for a course of five weeks, then a rest period of four weeks was given, before a second series of similar injections was begun numbering nine and extending over about six weeks. Prior to resuming the injections following the rest period, a half-dose was administered subcutaneously as a safeguard against a possible hypersensitivity. No response was noted and the full dosage was then resumed. Seven days after the final injection an 8-liter bleeding was taken and the antiserum yield was preserved with merthiolate in a 1:10,000 final concentration before shipment to Boyce Thompson Institute.

*Precipitin tests with mixtures of virus and homologous antiserum.* The quantitative serum-precipitin reaction described in detail previously (3, p. 389-393) was used to determine the ability of the three preparations to combine with and precipitate antibody. In brief, known concentrations of the virus preparations were mixed with varying volumes of the antiserum and the total volume in each tube was brought up to 5 cc. with physiological (0.85 per cent) saline. The tubes were placed in a water-bath at about 46° C. for two hours, during which period the contents of the tubes were kept constantly in motion by convection currents. The tubes were then held in a refrigerator for 48 to 72 hours and then transferred to a room at a temperature below freezing for several days. The contents of the tubes were thawed, the precipitates were centrifuged down and washed three times with chilled saline to remove extraneous matter. The entire procedure of washing the precipitates was conducted largely in a room where the temperature was below freezing and the rest of the time the tubes were kept packed in chipped ice to avoid a possible dissociation of virus and antibody. Upon completion of the washing, the total amounts of nitrogen in the precipitates were determined by the micro-Kjeldahl method and translated into terms of protein by multiplication with 6.25. The supernatant fluids obtained from the first decantation were mixed separately with antiserum and virus in order to ascertain whether either component was present in excess and, if so, which one. The results of the precipitin tests on the supernatant liquids indicated in which of the three zones of reaction a given test fell, whether in the region of antiserum excess, in the equivalence zone in which no excess virus or antiserum is demonstrable in the supernatant fluid, or in the region of antigen (virus) excess. If the tests were included in the first two zones, it was possible to determine the amount of antibody in the precipitate by subtracting the amount of antigen added from the total protein precipitated.

TABLE II

DATA ON THE PRECIPITIN REACTION BETWEEN INCREASING AMOUNTS OF  
TOBACCO-MOSAIC VIRUS, PREPARATION NO. 3 AND 1.0 CC. OF  
THE HOMOLOGOUS HORSE ANTISERUM†

Test No.	Total virus added, mg.	Total protein precipitated, mg.	Antibody protein by difference (col. 3-col. 2), mg.	Excess antibody or virus in supernatant as determined by serum-precipitin reaction	Original volume of antiserum used in tests, cc.††
1	0.030	0.138 0.161	0.108 0.131	Antibody	12
2	0.060	0.231 0.256	0.171 0.196	Antibody	6
3	0.096	0.354 0.372 0.398	0.258 0.276 0.302	Antibody	4
4	0.109	0.395 0.397 0.416	0.286 0.288 0.307	Antibody	4
5	0.130	0.432 0.455	0.302 0.325	Antibody	4
6	0.172	0.557 0.572 0.580	0.385 0.400 0.408	Antibody	3
7	0.258	0.723 0.725 0.749	0.465 0.467 0.491	Antibody	2
8	0.516	1.092 1.090 1.073	0.576 0.574 0.557	Antibody	1
9	0.901	1.741 1.737	0.840 0.836	Antibody	1
10	0.962	1.918 1.992 1.972	0.956 1.030 1.010	Antibody	1
11	1.092	1.992 1.921 1.947	0.900 0.829 0.855	Antibody	1
12	2.082	3.270 3.245	1.188 1.163	None	1
13	3.123	4.427 4.343 4.375	1.304 1.220 1.252	None	1
14	8.512	9.414 9.378 9.344	0.902 0.866 0.832	None	0.5

† The regression equation used in Figure 1 was calculated from the data in this table.

†† The amount of virus and antiserum used in the tests is expressed in this table in terms proportionate to 1.0 cc. of antiserum.

The results were tabulated and a graph was prepared by the method found to represent as near a linear relationship as possible between the amounts of virus added and the total amounts of protein precipitated,

TABLE III  
DATA ON THE PRECIPITIN REACTION BETWEEN INCREASING AMOUNTS OF  
TOBACCO-MOSAIC VIRUS, PREPARATIONS NOS. 2, 3, AND 4 AND  
1.0 CC. OF THE HOMOLOGOUS HORSE ANTISERUM†

Test No.	Virus prep. No.	Total virus added, mg.	Total protein precipitated, mg.	Antibody protein by difference (col. 4-col. 3), mg.	Excess antibody or virus in supernatant as determined by serum-precipitin reaction	Original volume of antiserum used in tests, cc. ††
1	2	0.090	0.345 0.341 0.348	0.255 0.251 0.258	Antibody	4
2		0.130	0.395 0.403 0.408	0.265 0.273 0.278	Antibody	4
3		0.169	0.543 0.544	0.374 0.375	Antibody	3
4		0.245	0.695 0.705	0.450 0.460	Antibody	0.5
5		0.253	0.729 0.717 0.726	0.476 0.464 0.473	Antibody	2
6		0.507	1.195 1.144 1.195	0.688 0.637 0.688	Antibody	1
7		0.896	1.940 1.902 1.915	1.044 1.006 1.019	Antibody	1
8		1.928	2.828 2.828	0.900 0.900	Antibody	0.5
9		1.960	2.664 2.664	0.704 0.704	Antibody	0.5
10		2.082	3.058 3.046 3.043	0.976 0.964 0.961	None	1
11		2.892	4.048 4.150	1.156 1.258	None	0.5
12		3.123	4.125 4.161 4.086	1.002 1.038 0.963	None	1

TABLE III—(Continued)

Test No.	Virus prep No.	Total virus added, mg.	Total protein precipitated, mg.	Antibody protein by difference (col. 4—col. 3), mg.	Excess antibody or virus in supernatant as determined by serum-precipitin reaction	Original volume of antiserum used in tests, cc.††
1	3	0.254	0.671 0.663 0.629	0.417 0.409 0.375	Antibody	2
2		0.508	1.068 1.065 1.068	0.560 0.557 0.560	Antibody	1
3		2.030	2.670 2.690 2.702	0.640 0.660 0.672	Antibody	0.5
1	4	0.244	0.643 0.636 0.635	0.399 0.392 0.391	Antibody	2
2		0.488	0.996 0.998 0.997	0.508 0.510 0.509	Antibody	1
3		1.952	2.596 2.576 2.488	0.644 0.624 0.536	Antibody	0.5

† The data in this table are represented in Figure 1 but were not used in calculating the regression equation.

†† See corresponding footnote in Table II.

when a constant volume (1.0 cc.) of antiserum was mixed with varying amounts of virus antigen. The data obtained, using the No. 3 antigen (Table II), were used to calculate the linear regression equation by the method of least squares (6, p. 134), when the logarithms of the amounts of Kjeldahl protein precipitated (*Y*-axis) were plotted on the logarithms of the amounts of virus added to a constant volume of undiluted antiserum (*X*-axis). Additional serum-precipitin tests were made in a single experiment with the Nos. 3 and 4 preparations as antigens, and in the case of the No. 2 preparation, several tests were made over a period of five months. The results of these experiments have been recorded in Table III and graphically in Figure 1. In Table IV a comparison has been made of the variances of the total amounts of Kjeldahl protein obtained experimentally from mixtures of the three virus preparations and antiserum (Table III) and the amounts calculated from the regression equation derived from the data pertaining to the No. 3 antigen (Table II). The ratios of these variances to that of the No. 3 preparation were used to determine significance.

## RESULTS OF SERUM-PRECIPITIN TESTS

Where only three determinations were made in a single experiment, using virus preparations Nos. 3 and 4 as antigens, the total amounts of precipitate formed in virus-antiserum mixtures showed a highly significant variance from the values calculated from the regression equation derived from the data obtained from tests with the No. 3 preparation (Table IV).

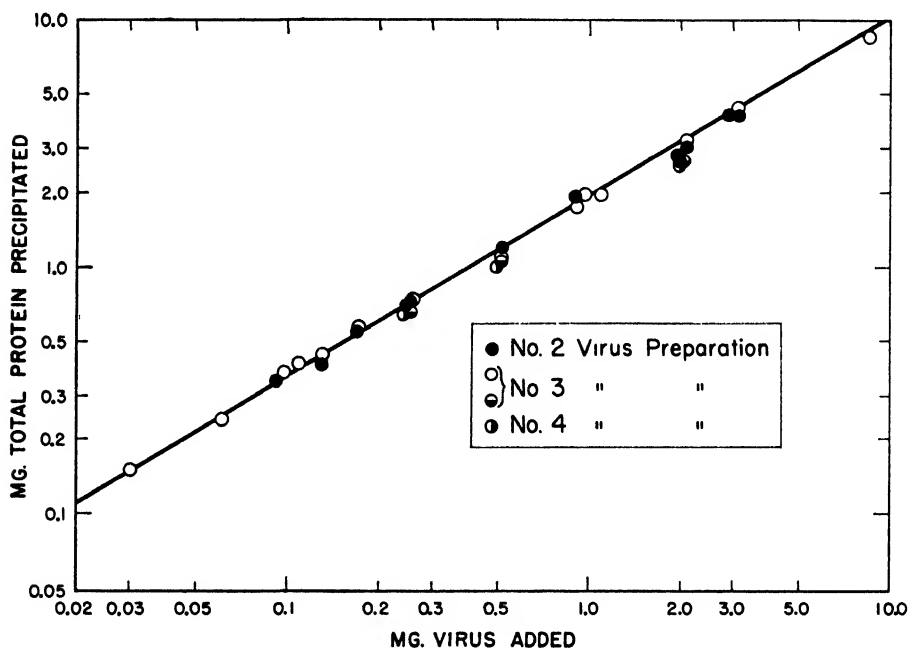


FIGURE 1. Relationship between mg. virus added and amounts of precipitate formed when increasing quantities of virus are added to a constant amount of antiserum. Regression of logarithm of mg. total protein precipitated on logarithm of mg. virus added.  $Y = 0.7217X - 0.1585$  for  $\circ$ , No. 3. Data for  $\bullet$ , No. 2,  $\odot$ , No. 3, and  $\bullet$ , No. 4 were not used in calculating the regression equation (see footnote† in Tables II and III).

In the case of the No. 2 preparation, this variance was significant at odds of 19:1, but when a correction was made for a 1.5 per cent contamination of the preparation with normal protein, found to be present, the variance was not significant (Table IV, footnote\*).

## DISCUSSION

Any variations in the results of the infectivity tests may be due to differences in the sensitivity of the groups of plants used for the separate tests, as well as the number of leaves inoculated.

The No. 3 virus preparation migrated as a single component when subjected to electrophoresis thus fulfilling the requirements for purity by this

method of testing. The No. 3 preparation, also the most infective of the three, thus proved to be the best source of virus for the production of specific antibody, since no antibody to non-virus protein would be produced in the antiserum. The No. 2 sample was too small in amount to permit an

TABLE IV

COMPARISON OF THE EXPERIMENTAL VALUES OBTAINED FOR THE TOTAL PROTEIN PRECIPITATED IN MIXTURES OF VIRUS PREPARATIONS NOS. 2, 3, AND 4 AND HOMOLOGOUS ANTISERUM WITH THOSE CALCULATED FROM AN EQUATION DERIVED FROM THE DATA ON PREPARATION NO. 3

Mg. total protein precipitated in mixtures of antiserum and virus preparations				Theoretical values obtained from logarithmic regression equation, $Y = 0.7217X - 0.1585\ddagger$			
No. 3†	No. 2††	No. 3††	No. 4††	No. 3	No. 2	No. 3	No. 4
0.150	0.345	0.654	0.638	0.153	0.339	0.717	0.696
0.244	0.402	1.067	0.997	0.253	0.442	1.182	1.149
0.375	0.544	2.687	2.553	0.355	0.534	3.212	3.123
0.403	0.700			0.389	0.699		
0.444	0.724			0.442	0.715		
0.570	1.178			0.541	1.180		
0.732	1.919			0.725	1.780		
1.085	2.664			1.195	3.132		
1.739	2.828			1.787	3.095		
1.953	3.040			2.054	3.272		
1.961	4.090			1.874	4.147		
3.258	4.124			3.272	4.384		
4.382				4.384			
9.379				9.039			
Variance				0.011504	0.030190	0.146410	0.175684
Ratio other variances to variance in column 5					3.4*	12.7*	15.3*

† Average of duplicate or triplicate determinations (see Table II, column 3).

†† Average of duplicate or triplicate determinations (see Table III, column 4).

‡ Regression of total amount of protein precipitated ( $Y$ -axis) on mg. virus added ( $X$ -axis).

\* Significant at odds of 19:1 or better, requiring a ratio of 6.5 or greater for columns 7 and 8, and 3.3 for column 6. If a correction is made for 1.5 per cent normal protein in the No. 2 virus preparation, the ratio is 2.8 for column 6, which is not significant (5).

electrophoretic test for purity so that the amount of normal-tobacco protein contaminating the virus nucleoprotein was determined by titration with antiserum to normal protein. Since the antiserum contained no antibody to normal-tobacco protein, the normal protein contaminating the No. 2 preparation would not be brought down with the virus-antibody precipitate but would remain in the supernatant fluid. Therefore, a correction was applied to the amounts of Kjeldahl protein used as antigen, which allowed for a contamination of the No. 2 sample with 1.5 per cent normal protein, before the values for total protein precipitated were calculated according to the regression equation, as indicated in Table IV, footnote\*.

The data obtained with the No. 2 virus preparation were quite comprehensive, including five separate experiments made over a period of five months and almost equalling in number the tests made with the No. 3 sample. The infectivity of the latter was found to be approximately seven times that of the former, so that these two virus preparations provided an excellent means of investigating the relationship between virus infectivity and ability to combine with and precipitate specific serum antibody.

No attempt was made to correct the No. 4 preparation of virus nucleoprotein for the presence of contaminating non-virus protein, since considerable denaturation of protein had evidently occurred during the electrophoretic tests and there was the possibility that some of the virus protein might also have become denatured.

Three precipitin tests in a single experiment, as evidenced by the results obtained even when the standard virus sample (No. 3) was used, are inadequate to make a fair comparison between the variances of the observed amounts of protein in the precipitate and those calculated from the regression equation.

The lack of close agreement between duplicates and triplicates in some cases regarding the total amounts of protein precipitated from virus-antiserum mixtures (Tables II and III) is believed to be due to the large size of the rod-shaped virus particle which may occasionally obstruct contact between antibody and the reactive groups of the antigen.

#### SUMMARY AND CONCLUSIONS

1. A brief discussion is given of the literature dealing with the ability of plant viruses to react with specific serum antibody after having been rendered non-infectious by various physical and chemical treatments.

2. The method of virus preparation and the test for purity by subjection to electrophoretic separation are described.

3. The method of determining the relative infectivity of the virus preparations by inoculation on *Nicotiana glutinosa* L. as well as the subsequent statistical analysis of the results are given.

4. The preparation of virus antiserum is described, also the serum-precipitin reaction which serves as a means of determining the relative ability of the virus preparations to react with specific antibody on a quantitative basis.

5. The results are discussed and the conclusion is drawn that virus preparations differing greatly in infectivity may react similarly with specific antibody on a quantitative basis.

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# RESPONSE AND RECOVERY OF DANDELION AND PLANTAIN AFTER TREATMENT WITH 2,4-D

A. E. HITCHCOCK AND P. W. ZIMMERMAN

Results of weed killing tests with 2,4-D (2,4-dichlorophenoxyacetic acid), carried out during 1945 and 1946, exhibited such a wide range of variability that the relative importance of several limiting factors could not be ascertained. In the case of tests on turf areas, a marked and lasting discoloration occurred in some instances and not in others, with no consistent relation to the response of non-grass weeds to the 2,4-D treatment.

Variability in results with 2,4-D occurred also in non-turf areas. In either or both turf and non-turf areas the following plants were not eradicated, although parts or all of the tops of some plants may have been killed:

<i>Common name</i>	<i>Specific name</i>
Joe-pye weed	<i>Eupatorium purpureum</i> L.
Wild strawberry	<i>Fragaria virginiana</i> Duchesne
Wild lettuce	<i>Lactuca canadensis</i> L.
Wild snapdragon	<i>Linaria vulgaris</i> Hill
Oxalis	<i>Oxalis</i> sp.
Solomon's seal	<i>Polygonatum biflorum</i> (Walt.) Ell.
Cinquefoil	<i>Potentilla</i> sp.
Virginia creeper	<i>Pseodera quinquefolia</i> (L.) Greene
Poison ivy	<i>Rhus toxicodendron</i> L.
Wild blackberry	<i>Rubus allegheniensis</i> Porter
Maple	<i>Acer</i> sp.
Yarrow	<i>Achillea millefolium</i> L.
Wild onion	<i>Allium vineale</i> L.
Common milkweed	<i>Asclepias syriaca</i> L.
Aster	<i>Aster</i> sp.
Wild black birch	<i>Betula lenta</i> L.
Mouse-ear chickweed	<i>Cerastium vulgatum</i> L.
Canadian thistle	<i>Cirsium arvense</i> (L.) Scop.
Horsetail	<i>Equisetum arvense</i> L.
Ash	<i>Fraxinus americana</i> L.
Japanese honeysuckle	<i>Lonicera japonica</i> Thunb.
Carpetweed	<i>Mollugo verticillata</i> L.
Common knotweed	<i>Polygonum aviculare</i> L.
Smartweed	<i>Polygonum</i> sp.
Purslane	<i>Portulaca oleracea</i> L.

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Wild cherry	<i>Prunus</i> sp.
Locust	<i>Robinia pseudoacacia</i> L.
Wild rose	<i>Rosa</i> sp.
Wild raspberry	<i>Rubus aculeatissimus</i> (C. A. Mey.) Regel & Tiling
Sheep's-sorrel	<i>Rumex acetosella</i> L.
Dock	<i>Rumex patientia</i> L.
Sassafras	<i>Sassafras variifolium</i> (Salisb.) Ktze.
False Solomon's seal	<i>Smilacina racemosa</i> (L.) Desf.
Green brier	<i>Smilax</i> sp.
Goldenrod	<i>Solidago</i> sp.
Grass-leaved chickweed	<i>Stellaria graminea</i> L.
Common chickweed	<i>Stellaria media</i> (L.) Cyrill
Common mullein	<i>Verbascum thapsus</i> L.
Speedwell	<i>Veronica</i> sp.
Wild violet	<i>Viola papilionacea</i> Pursch.

These results are in general agreement with those in the Report of The Policy Committee on Herbicides for 1947 (5) in which most of the species just mentioned are classified as intermediate or resistant in susceptibility to 2,4-D treatment. The possible exceptions are cinquefoil and mouse-ear chickweed which in The Policy Committee Report (5) are listed as susceptible, although qualified by the statement (p. 6): "Tops readily killed by 2,4-D at some stages, roots frequently killed by one application." Dandelions and plantains were also covered by this statement. The first ten species listed above were reported previously as surviving in plots of Japanese honeysuckle treated with 0.1 to 0.2 per cent 2,4-D (3). These particular ten species were not the only ones which survived in the 2,4-D treated honeysuckle plots.

During 1946 and the spring of 1947 extensive tests with 2,4-D were carried out on turf and non-turf areas under a wide range of conditions. Concentrations of 2,4-D ranged from 0.02 to 2.5 per cent and rates of application from 1 gallon per 360 square feet to 1 gal. per 30 sq. ft. A summary report describing the relative resistance of established stands of Japanese honeysuckle to treatment with 2,4-D has already been given (3). The present report deals primarily with the effectiveness of 2,4-D for eradicating dandelion and plantains. Recovery responses of dandelion in treated plots, including new seedlings and rosettes from old roots which had not been entirely killed, constitute a major part of this report. Although plantains were generally eradicated by a single treatment with 2,4-D, the prevalent belief that dandelions also can be eradicated with a single treatment is not supported by the present results. In contrast to the results with dandelions, there was little or no regrowth of narrow-leaved and two species of broad-leaved plantains in treated plots.

## MATERIALS AND METHODS

Spray solutions of 2,4-Dow Weed Killer containing 20 per cent 2,4-dichlorophenoxyacetic acid equivalent were used except when otherwise noted. In Experiments No. 5 and 6 a crystalline preparation of 2,4-D obtained from The Dow Chemical Company, Midland, Michigan, was dissolved in the carrier. The spray solution in all cases was applied by means of a Hudson Junior sprayer of two-gallon capacity. A measured quantity of the solution was delivered on a known area in all of the tests. A standard treatment consisted of 0.1 per cent 2,4-D applied at the rate of 1 gal. per 180 sq. ft. This falls within the medium range recommended by The Policy Committee on Herbicides (5). Scott's dust preparation was applied with a spreader as recommended by the manufacturer.

The names of plants referred to in the present tests are as follows:

<i>Common name</i>	<i>Specific name</i>
Creeping bent	<i>Agrostis</i> sp.
Crab grass	<i>Digitaria sanguinalis</i> (L.) Scop.
Fescue grass	<i>Festuca</i> sp.
Hawkweed (yellow)	<i>Hieracium</i> sp.
Rye grass (perennial)	<i>Lolium perenne</i> L.
Plantain	<i>Plantago lanceolata</i> L. ✓
Plantain	<i>Plantago major</i> L. ✓
Plantain	<i>Plantago rugelii</i> Dene. ✓
Annual bluegrass	<i>Poa annua</i> L.
Kentucky bluegrass	<i>Poa pratensis</i> L.
Rough bluegrass	<i>Poa trivialis</i> L.
Patience dock	<i>Rumex patientia</i> L.
Dandelion	<i>Taraxacum officinale</i> Weber
White clover	<i>Trifolium repens</i> L.

## RESPONSE OF DANDELION AND PLANTAIN TO 2,4-D

## EXPERIMENT I

The lawn area selected for these tests contained many dandelions in addition to many weeds of other species, including chickweeds and speedwells. Grasses consisted mainly of *Poa annua*, *P. trivialis*, and creeping bents. Kentucky bluegrass was present in a few scattered areas constituting less than 25 per cent of the grasses. There was a considerable amount of white clover in all plots. The soil was of relatively low fertility due to lack of a regular fertilizer and top dressing program during recent years. Treated plots 9×20 feet were alternated with non-treated control plots of the same area, the entire area being mowed weekly.

*Results.* The highest concentration of 2,4-D (0.2 per cent) was the most effective of the six treatments for killing dandelion tops, and was the only

treatment which killed the tops of all plants (Table I). The other five treatments were about equally effective. Recovery responses during 1946 and up to April 1947 decreased with increasing concentration and increasing rates of application but the differences were not great (Table I). By September 1946, and also in April 1947, relatively large numbers of dandelions were again present in all treated plots, resembling in appearance the dandelions in the non-treated control plots. A count was not made at this time. It could not be determined with certainty which dandelion crowns represented a recovery growth from well established roots and which ones may have developed from new seedlings. All six of the treatments administered in May were highly effective in reducing or preventing the flowering of dandelions in September. In contrast to the abundant flowering of dandelion in all six non-treated control plots, there was little or none in the treated plots.

TABLE I

RESPONSE OF DANDELION IN GRASS PLOTS 180 SQ. FT. IN AREA SPRAYED WITH 2,4-D MAY 1, 1946. NON-TREATED CONTROL PLOTS OF SAME AREA ALTERNATED WITH TREATED PLOTS

Type of observation and date	Per cent 2,4-D applied at rate of 1 gal./180 sq. ft.			Per cent 2,4-D applied at different rates (sq. ft./gal.)		
	0.05	0.10	0.20	360	180	90
Estimated per cent kill of tops, June 5, 1946	> 90	> 90	100	> 90	> 90	> 90
Number of plants, June 28, 1946	8	10	4	12	19	10
Number of flowers, Sept. 19, 1946	3	2	0	3	0	0
Estimated number of plants, April 1, 1947	210	189	126	252	168	210

In addition to killing the tops of more than 90 per cent of the dandelions, all six treatments caused a noticeable and lasting discoloration of the plots. The discoloration increased noticeably with increasing concentration and rate of application. Much of the initial discoloration was caused by dying leaves of white clover and to the exposed clippings normally hidden by intact leaves of white clover. Injured and dead leaves of dandelion, creeping bent, *Poa trivialis*, chickweed, and speedwell also contributed to the discoloration. Bare areas caused by the disappearance of dandelion crowns were filled in by crab grass during the summer of 1946. The luxuriant growth of crab grass in treated plots was more extensive than in the intervening control plots. Conversely, the growth of desirable lawn grasses was much more extensive in the control plots during the fall of 1946 and throughout the spring of 1947.

During the spring of 1947 the extensive bare areas, occupied the previous fall by crab grass, filled in slowly with chickweed, speedwell, dandelion,

and plantain. Lack of a complete covering of plants, together with the persistent yellowish discoloration of the basal portions of *Poa trivialis* caused the treated plots to be clearly outlined throughout the spring of 1947. There was also noticeably less clover in the treated as compared with the control plots. By June 1947, the three plots which received different concentrations of 2,4-D were still outlined, whereas the three plots which had received different rates of application had recovered sufficiently to make their location difficult without reference to the plot markers. These differences in discoloration existed, notwithstanding that equivalent quantities of 2,4-D were applied to the same unit area in both series of tests (0.05 per cent at 1 gal. per 180 sq. ft. equivalent to 0.1 per cent at 1 gal. per 360 sq. ft., 0.2 per cent at 1 gal. per 180 sq. ft. equivalent to 0.1 per cent at 1 gal. per 90 sq. ft., etc.).

#### EXPERIMENT 2

The lawn area selected for these tests consisted of well established thick turf in which Kentucky bluegrass was the principal grass. Weeds, including dandelion and narrow-leaved plantain, were scattered throughout the treated and control plots. Sixteen of the 18 treated plots contained both dandelion and plantain and two plots contained dandelion but no plantain. Treated plots (3×3 feet) were alternated in checkerboard fashion with non-treated control plots of the same area.

*Results.* The tops of all of the dandelion and plantain in treated plots were killed within a period of five weeks without causing lasting discoloration of the area under test. During the remainder of 1946 only one dandelion and one plantain appeared in the treated plots. In April 1947 there were no plantains in any of the 16 treated plots. On April 22, 1947, there was a total of 11 dandelions in 5 of the 18 treated plots, and on June 20 there were 49 in 15 of the 18 plots. The plots which had received a single application of 2,4-D on July 17, 1946, contained the largest numbers of dandelions the following year. The sprays applied July 17 were less effective for eradicating dandelion than the same sprays applied July 3 or the sprays applied on both of these dates. Narrow-leaved plantain was eradicated in 15 of 16 plots by sprays applied on either date. Considering the greater effectiveness of the sprays applied July 3, as compared with the same sprays applied July 17, any additional influence of respraying on dandelion could scarcely be expected and was not found in these tests.

The rate of application was not critical in the range 1 gal. per 90 to 270 sq. ft. for the sprays applied on July 3, 1946, inasmuch as all dandelion and plantain tops were killed by the lowest rate (1 gal. per 270 sq. ft.). There was possibly a slight influence of the rate of application in the case of sprays applied July 17, 1946, particularly if judged on the basis of the number of dandelions present on April 22, 1947 (column 4, Table II).

The lesser number of dandelions in the plot receiving the highest rate (1 gal. per 90 sq. ft.) holds also for the later counts made on June 20, 1947 (column 5, Table II). Presumably the conditions which existed on July 17 were such that the lowest rate of 1 gal. per 270 sq. ft. represented more

TABLE II  
INFLUENCE OF RATE OF APPLICATION OF 0.1 PER CENT 2,4-D AND OF RESPRAYING  
ON THE ERADICATION OF DANDELION AND NARROW-LEAVED  
PLANTAIN IN WELL ESTABLISHED TURF

Dates sprayed, 1946	Rate, sq. ft./gal.	No. of dandelion			No. of plantain		
		July 3, 1946	Apr. 22, 1947	June 20, 1947	July 3, 1946	Apr. 22, 1947	June 20, 1947
July 3	270	2	0	0	11	0	0
	270	10	0	1	—	—	—
	180	6	0	0	6	0	0
	180	4	0	1	2	0	0
	135	9	0	2	12	0	0
	108	8	0	0	12	0	0
	90	6	0	1	12	0	0
	Av.		0.0	0.7		0.0	0.0
July 17	270	12	3	8	8	0	0
	270	8	3	6	25	0	3
	180	3	2	2	4	0	0
	180	9	0	6	26	0	0
	135	22	3	6	16	0	0
	108	10	0	5	7	0	0
	90	10	0	2	—	—	—
	Av.		1.6	5.0		0.0	0.5
July 3	270						
" 17	270	16	0	1	24	0	0
" 3	180						
" 17	270	16	2	2	30	0	0
" 3	270						
" 17	180	8	0	1	9	0	0
" 3	180						
" 17	180	5	0	5	16	0	0
	Av.		0.5	2.3		0.0	0.0

nearly the minimum effective rate for killing than was the case for the same spray applied on July 3.

### EXPERIMENT 3

The area selected for these tests consisted of five mowed and five non-mowed plots (3×3 feet) on an abandoned tennis court where there was a sparse growth of grass in addition to dandelion and plantain. The area was shaded after 2 o'clock by a nearby building.

*Results.* Tops of dandelion and plantain were killed within five weeks after treatment in all five mowed plots and in three non-mowed plots. The initial killing and the lasting effects of the single sprays were slightly

greater for the higher rate of 1 gal. per 90 sq. ft., being most pronounced for dandelion in the mowed plots. However, the lasting effects of all five treatments on dandelion were considerably greater in the non-mowed

TABLE III  
INFLUENCE OF RATE OF APPLICATION OF 0.1 PER CENT 2,4-D, OF RESPRAYING,  
AND OF WEEKLY MOWING OF PLOTS ON TOP KILL AND RECOVERY  
OF DANDELION AND BROAD-LEAVED PLANTAIN

	Rate, sq. ft./gal.	Date sprayed	% Top kill after weeks			No. of plants		
			3	5	8	Before spraying	Sept. 19, 1946	April, 1947
Dandelion								
Mowed weekly	180	July 12	33	100	67	3	10	7
	90	" 12	75	100	75	8	5	4
	90	" 26	94	—	100	16	8	5
		" 12	83	100	100	6	10	7
	180	" 19						
		" 12	100	100	80	5	5	5
Av.	180	" 26	77	100	84		7.6	5.6
Not mowed	180	" 12	77	92	77	13	1	2
	90	" 12	77	100	92	13	6	0
	90	" 26	92	—	83	12	4	2
	180	" 12	100	100	100	8	0	0
		" 19						
	180	" 12	83	100	100	18	5	6
Av.		" 26	86	98	90		3.2	2.0
Plantain								
Mowed weekly	180	July 12	61	100	100	18	10	6
	90	" 12	76	100	100	17	1	0
	90	" 26	57	—	100	14	2	0
	180	" 12	81	100	100	36	8	0
		" 19						
	180	" 12	100	100	100	10	2	0
Av.		" 26	75	100	100		4.6	1.2
Not mowed	180	" 12	50	85	85	20	3	0
	90	" 12	40	100	100	5	0	0
	90	" 26	20	—	70	10	1	0
	180	" 12	40	100	100	5	0	0
		" 19						
	180	" 12	31	100	100	16	0	0
Av.		" 26	36	96	91		0.8	0

plots as judged by the smaller number of plants in April 1947 (Table III). Plantains were eradicated in 9 of 10 plots by April 1947, whereas dandelions were eradicated in only 2 of 10 plots by the same date.



## EXPERIMENT 4

The test area was located in a different part of the abandoned tennis court used for treatments in Experiment 3. Treated plots in this experiment were in a fairly good turf area consisting mainly of Kentucky bluegrass which for more than ten years had received the lime wash from an adjacent regularly used tennis court. Nine plots were mowed weekly from July 12 to September 20, 1946, whereas nine similar plots were not mowed. After the results had been obtained, relating to the rate of killing the tops of all dandelions, the nine mowed plots were sprayed again on September 20, 1946, for the purpose of determining residual effects of 2,4-D on germination and early seedling growth of lawn grass. Three of the nine mowed and three of the non-mowed plots were top dressed and seeded 14 days later (October 4, 1946). Data for these three plots appear in lines 1, 4, and 7 of the body of Table IV. The intention was to treat the remaining plots in a similar manner at later periods. Since there was no delay in germination or in early seedling growth of the grass, the residual phase of this experiment was terminated, but the observations on these plots were continued as indicated in part B of Table IV.

*Results.* The rate of killing of the tops of all dandelions was slightly more rapid in mowed plots, but not significantly different, as compared with the non-mowed plots (columns 6 and 7, Table IV). The rate of application

TABLE IV  
RESPONSE OF DANDELION THE SAME YEAR AND THE YEAR FOLLOWING  
TREATMENT WITH 0.1 PER CENT 2,4-D

Rate, sq. ft./gal.	Date sprayed, 1946		No. plants at start		Part A				Part B	
					Av. No. of weeks for tops of all plants to die		Plants present Sept. 19, 1946 (% of original number)		Plants present April, 1947 (% of original number)	
	First spray	Second spray	Mowed plots	Non- mowed plots	Mowed weekly	Not mowed	Mowed weekly	Not mowed	Mowed weekly	Not mowed
180	July 12	—	30	7	4	5	87	214	0	8
	" 19	—	26	24	5	7	46	58	4	50
	" 26	—	15	20	5	7	47	30	20	45
90	" 12	—	24	21	4	4	115	34	0	14
	" 19	—	34	28	5	7	76	54	3	36
	" 26	—	20	18	7	7	25	33	10	39
180 Respray	" 12	July 19	16	16	3	3	112	137	0	31
	" 12	" 26	31	12	5	4	90	92	0	58
	" 12	Aug. 2	23	19	3	5	100	47	9	42

was not critical in the range 1 gal. per 90 to 180 sq. ft. for the initial maximum killing of dandelion tops, but the respray treatments were significantly (at 5 per cent level) more effective. Likewise, the differences due to time of treatment were significant at the 5 per cent level, mainly on the basis of the higher effectiveness of the single sprays applied July 12, 1946, and the first respray applied July 19, 1946. Differences due to the kind of spray treatment and to the time of application were of the same order of magnitude.

Treatments which caused the most rapid killing of dandelion tops in mowed plots (column 6, Table IV) permitted the greatest recovery by September 19, 1946 (column 8, Table IV). The correlation for this relationship is  $r_r = .8667$  (4, p. 472). There was no similar close relationship for non-mowed plots in September ( $r_r = .4977$ ), but the recovery in April 1947 (column 11, Table IV) was correlated with the initial killing effects (column 7, Table IV) the previous year ( $r_r = .7500$ ). Although the respray applied to the nine mowed plots on September 20 was highly effective for the eradication of dandelions then present (column 8 vs. column 10, Table IV), there appeared to be an important lasting effect up to April 1947, of the initial spray treatments applied to these same plots during the period July 12 to August 2, 1946. The correlation for this relationship (columns 6 and 10, Table IV) is represented by  $r_r = .8405$ . This held true also for the non-mowed plots which were not resprayed in September (columns 7 and 11, Table IV). Part of the effect assigned to the time of applying the first spray may be due to top dressing and seeding. It seems unlikely that the major part of the effect occurring in 1947 can be attributed to top dressing, since the order of effectiveness for all nine treatments before (column 6, Table IV) and after (column 10, Table IV) top dressing is the same. The same held true for the non-mowed plots (columns 7 vs. 11, Table IV).

#### EXPERIMENT 5

All dandelion plots were located in the abandoned tennis court referred to in Experiments 3 and 4. Plantain plots No. 1 to 6 were located in an open field bordering an unimproved road. Plots No. 7 to 9 contained dandelions and plantains. Dandelion and plantain plots No. 1 to 6 (part A, Table V) were trimmed just before treatment, but not thereafter. Spray solutions of 2,4-D were made up in 2 per cent Tween #20, an emulsifying agent purchased from the Atlas Powder Company, Wilmington, Delaware. In the case of the 1 per cent solution of 2,4-D, the neutral equivalent of triethanolamine was used in addition to the Tween #20.

**Results.** The tops of all dandelions and plantains were killed by a 0.1 per cent 2,4-D spray applied once or twice (part A, Table V). Respraying with 0.1 per cent 2,4-D caused a more rapid killing of dandelion tops

TABLE V

INFLUENCE OF CONCENTRATION OF 2,4-D, TIME OF YEAR APPLIED, AND RESPRAYING ON KILLING OF TOPS OF DANDELION AND BROAD-LEAVED PLANTAIN

Plot No.	% 2,4-D	Dandelion								Plantain							
		Date sprayed		No. at start	% Top kill after days or date					Date sprayed		No. at start	% Top kill after days or date				
		First spray	Second spray		14	19	22	27	33	First spray	Second spray		22	32	44		
Part A																	
1	0.04	Sept. 4	4	Sept. 13	20	0	0	0	0	45	Sept. 5	5	Sept. 16	15	27	87	100
2	0.04	" 4	4	" 18	21	0	5	5	5	71	" 5	5	" 25	18	6	67	100
3	0.05	" 5	5	—	8	0	38	75	88	100	" 5	5	—	21	19	52	67
4	0.10	" 4	4	Sept. 13	14	14	71	86	100	100	" 5	5	Sept. 16	27	56	59	100
5	0.10	" 4	4	" 18	12	25	92	100	100	100	" 5	5	" 25	26	4	77	100
6	0.10	" 4	4	—	14	7	—	50	71	100	" 5	5	—	27	9	70	100
Part B																	
							Aug. 21, 1946		May 23, 1947						Aug. 21, 1946		May 23, 1947
7	0.04	June 17	—		8		12		37	June 17	—		5		60		100
8	0.20	" 17	—		20		93		95	" 17	—		6		100		100
9	1.00	" 17	—		22		100		45	" 17	—		3		100		100

than a single spray but there was no difference in the case of plantain. The lower concentration of 0.04 per cent applied twice killed all plantains but only 45 to 71 per cent of the dandelion tops. A 0.05 per cent 2,4-D spray applied on September 5, 1946, was as effective on dandelion, but less effective on plantain, as compared with a 0.1 per cent spray applied the previous day (September 4). The percentage and rate of killing during the first six weeks after treatment depended upon the species of plant, the concentration and rate of applying 2,4-D, and the date of treatment.

Data relating to the influence of concentration for the sprays applied June 17, 1946, are shown in part B of Table V. Maximum killing of the tops of dandelion and plantain within two months after treatment increased with increasing concentration of 2,4-D. The 0.2 per cent spray applied in June was less effective for the killing of dandelion tops than the 0.1 per cent spray applied in September. With respect to the final effects the following spring, concentrations of 2,4-D in the range 0.04 to 1.0 per cent applied in June 1946 were less effective for eradicating dandelion than the 0.04 and 0.1 per cent sprays applied in September 1946. The appearance of plot No. 9 at the time of treatment and ten days later is shown in Figure 1 and at later periods in Figure 2. The appearance of plot No. 9 on June 13, 1947, is shown in Figure 2 B. Of the 40 dandelions present in plot No. 9 at the time of photographing (Fig. 2 B), 17 were 10 to

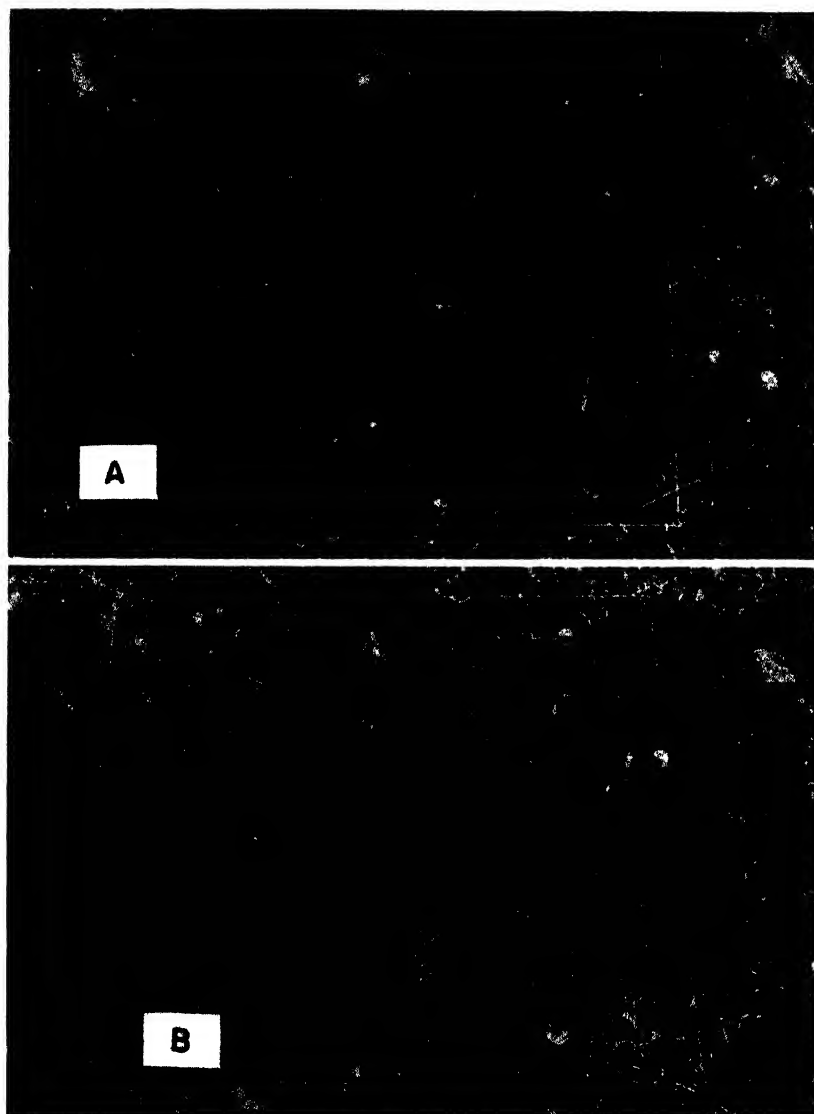


FIGURE 1. Dandelion plot June 17, 1946 (A) at time of applying 1.0 per cent 2,4-D in Tween #20-triethanolamine and (B) 10 days later showing initial curling and bending responses. Later responses shown in Figure 2.

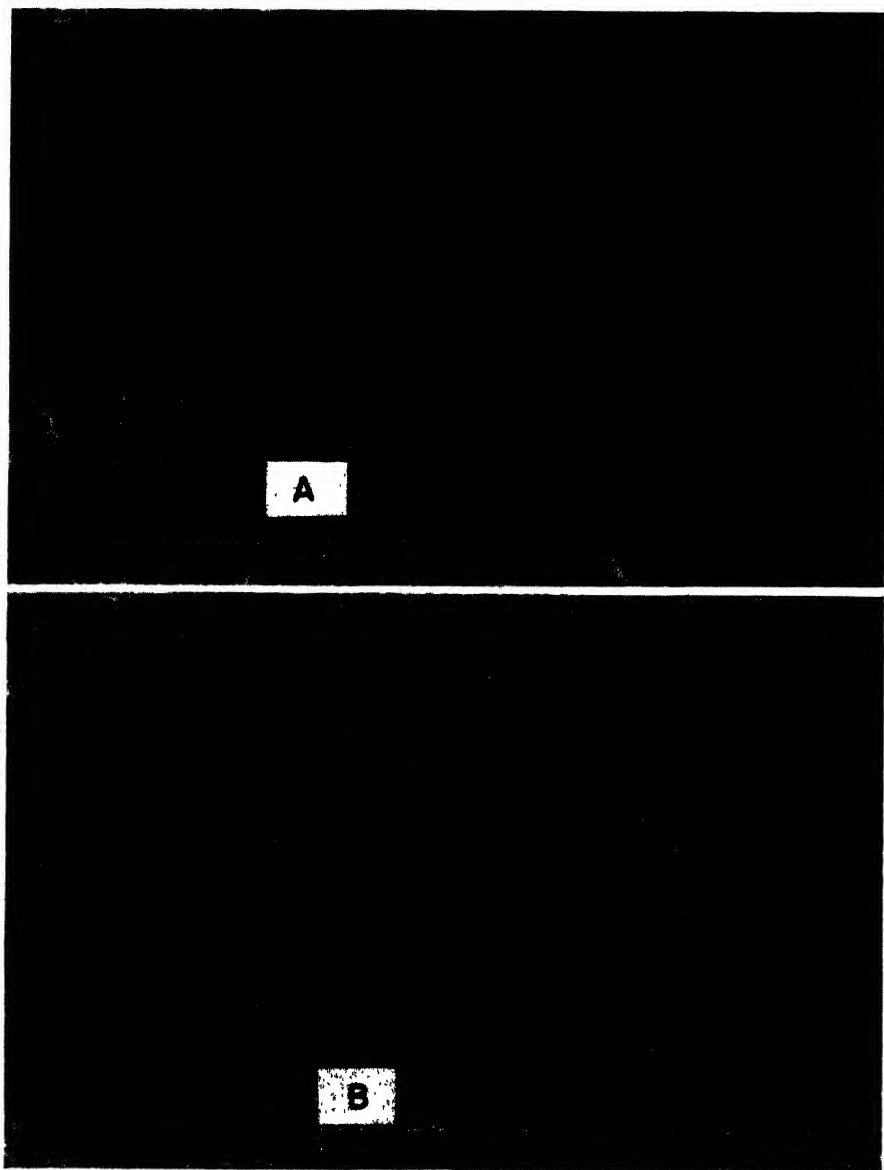


FIGURE 2. (A) Complete killing of tops of all dandelions and most other plants on July 24, 1946, resulting from 1.0 per cent 2,4-D spray applied June 17, 1946. (B) Same plot as in A showing regrowth one year later (June 13, 1947). Earlier appearance of plot shown in Figure 1.

12 inches in diameter, 12 had old fruit stalks still attached, and 9 had newly formed flower buds. Two plants not shown in Figure 2 B were removed for examination, one in September 1946, and one in April 1947. Both of these plants had several crowns, each with its etiolated stem-like base similar to those shown at the left in Figure 3B. This was true also for dandelion plants removed from other treated plots two months or more after treatment (Fig. 3). The branching habit of the plants removed from a plot treated with 0.1 per cent, 2,4-D (Fig. 3 B) was similar to that of plants removed from a control plot (Fig. 3 A). Since this characteristic branching (Fig. 3 and 5 A) follows flowering and is associated with an age of two years or more (6), it seems apparent that all of the dandelion plants are not killed entirely by 2,4-D even by an excessively high concentration such as 1.0 per cent.

## EXPERIMENT 6

Dandelions and two species of broad-leaved plantains of uniform size and appearance were transplanted April 18, 1946, in preparation for spraying three months later. The rows and the plants in each row were spaced one foot apart, so that the location of each plant was known throughout the course of the experiment. The area was mowed weekly.

In some of the earlier tests with 2,4-D the variability in response of plantain appeared to be due partly to a differential response of the two species, *Plantago major* and *P. rugelii*, the former being more sensitive.

TABLE VI

RESPONSE OF DANDELION AND TWO SPECIES OF BROAD-LEAVED PLANTAIN TO 2,4-D AND TO 2,4,6-TRICHLOROPHOXYACETIC ACID APPLIED AT THE RATE OF 1 GAL./180 SQ. FT.

Compound and concn.	Date sprayed, 1946	Dandelion						Plantain							
		No. plants	% Top kill after days				% Top kill May 16, 1947	No. plants	Species of <i>Plan-ago</i>	% Top kill after days				% Top kill May 16, 1947	
			14	31	66	78				14	31	66	78		
2,4-D, 0.1%	July 15	8	25	87	63	50	50	4	<i>rugelii</i>	0	75	75	100	100	
			4	<i>major</i>	100	100	100	100	100						
	July 15 and 29	8	50	100	88	88	75	4	<i>rugelii</i>	25	100	100	100	100	
			4	<i>major</i>	100	100	100	100	100						
2,4,6-Cl <sub>3</sub> POA, 0.1%	July 15	8	0	0	0	0	0	4	<i>rugelii</i>	0	0	0	0	25	
			4	<i>major</i>	0	0	0	0	0	50					
	July 15 and 29	8	0	0	0	0	0	4	<i>rugelii</i>	0	0	0	0	0	
			4	<i>major</i>	0	0	0	0	0	50					

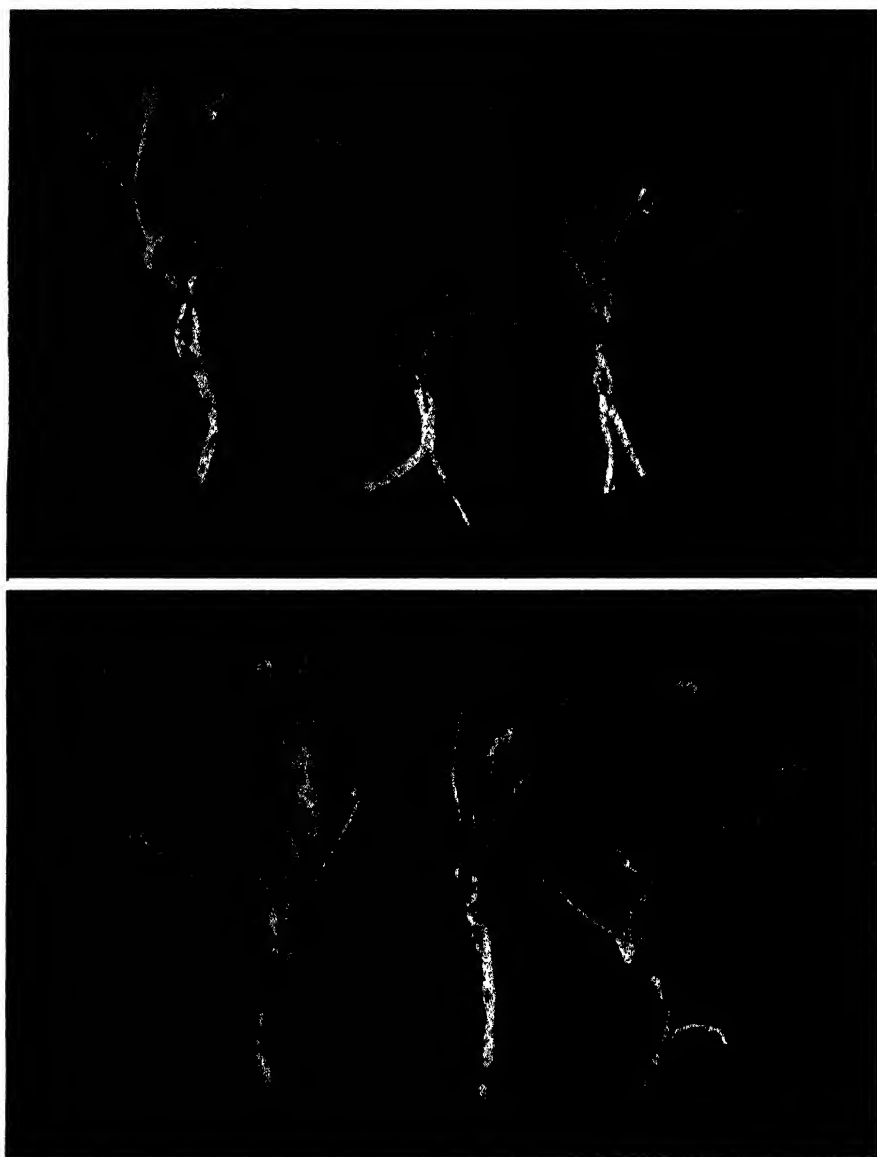


FIGURE 3. Dandelions removed June 12, 1947, from (A) control lawn plot and (B) plot treated with 0.1 per cent 2,4-D September 16, 1946. Roots pruned for photographing. Note multicapital type of branching which indicates all plants are more than one year old. Root at right in B damaged by 2,4-D, permitting growth of only one rosette.

The present tests were carried out to determine this point for 2,4-D and also for 2,4,6-trichlorophenoxyacetic acid which had proved in previous field and greenhouse tests to be ineffective for killing dandelion and plantain, although causing modification of plantain leaves.

*Results.* Data in Table VI show that a single application of 0.1 per cent 2,4-D was not effective in killing the tops of all dandelions, whereas the application of two sprays was. Both spray treatments were not sufficiently effective to prevent the recovery of at least a few of the plants the same year (1946) and the following spring (1947). The percentage recovery in 1947 was 50 in the plots receiving a single spray and 25 in the plots receiving two sprays. Recovery of the three dandelions was definitely from roots of plants showing top kill 31 days after treatment. In addition to having a record of the specific location of each plant, the rosettes which later grew from such plants were characteristic of dandelions two or more years old. None of the dandelions was completely killed by 2,4,6-trichlorophenoxyacetic acid.

In contrast to the results obtained with dandelion, the 2,4-D sprays killed all plantains in 2 to 11 weeks, and there was no recovery by the following May 1947. The rate of killing *Plantago major* was more rapid than for *P. rugelii* and the respray treatment caused a more rapid killing than a single spray.

Plantains sprayed with 2,4,6-trichlorophenoxyacetic acid were not killed up to 78 days after treatment although in many cases the new leaves were modified and stunted. By May 1947, 50 per cent of the *Plantago major* and 13 per cent of *P. rugelii* were killed. In contrast to 2,4-D, the respray with 2,4,6-trichlorophenoxyacetic acid was not more effective than a single spray.

The greater resistance of *Plantago rugelii* to weed killers of the 2,4-D type is of practical interest because it is the predominating species in this locality.

#### EXPERIMENT 7

A solid somewhat granular preparation of 2,4-D was applied to two lawns which differed with respect to soil moisture and drainage, and to the proportion of grass species. One lawn area was part of that used for tests described in Experiment 2. This particular lawn was well drained, exposed to full sunlight all day, and consisted of a well established turf containing about 50 per cent Kentucky bluegrass, and considerable amounts of fescue grasses and white clover. Dandelions were scattered throughout the lawn, whereas narrow-leaved plantains occurred in scattered patches. There were relatively few broad-leaved plantains. This lawn was mowed closely every four to five days.

The other lawn was in a low lying poorly drained area which was wet,



except during a drought, and remained soggy for 24 hours or more after an average rain of a few hours' duration. This area was exposed to full sunlight most of the day. There was less than 50 per cent Kentucky bluegrass, a high percentage of bent grass (presumably Colonial bent), and appreciable amounts of rye grass, *Poa trivialis*, annual bluegrass, and white clover. Dandelions and broad-leaved plantains were abundant throughout the lawn.

"Scotts Lawn Food plus Weed Control" was applied, as recommended by the manufacturer, at the rate of 5 pounds per 1,000 sq. ft., using a Scott's spreader No. 50 set at mark No. 4. Treatments were applied June 4, 1947, on a bright day (50 per cent relative humidity), and at a minimum temperature of 67° F. in the shade. The areas treated were 1,080 square feet and 1,280 square feet respectively on the well drained and poorly drained lawns.

*Results.* Within one month after treatment the tops of all but one dandelion and the tops of all plantains were killed in the well drained lawn. About 90 per cent of the foliage of white clover was killed within the first two weeks, but the recovery at the end of 30 days was estimated to be about 50 per cent. There was no lasting discoloration or permanent injury on any of the grasses. This treatment was effective in killing the tops of dandelions and plantains without causing undesirable or lasting discoloration.

In contrast to the effectiveness of Scott's 2,4-D powder on the well drained lawn, the results on the poorly drained lawn were extremely unsatisfactory. Although the tops of most plantain were killed, relatively few dandelion tops were killed. In some areas there were 100 or more dandelions per square yard. In addition to an ineffective killing of dandelions, scattered areas several feet in diameter were badly discolored. The cause of this discoloration could not be ascertained. Colonial bent appeared not to be noticeably injured. Presumably soil conditions must be regarded as an important limiting factor in determining the effectiveness of 2,4-D for killing dandelions in lawns.

#### RECOVERY OF DANDELION IN TREATED PLOTS

A standard treatment of 2,4-D consisting of a 0.1 per cent solution applied at the rate of 1 gal. per 180 sq. ft. generally killed the tops of most or of all dandelions and plantains within two to five weeks after treatment (Fig. 4 A and B and Tables I to VI). In plots where the tops of all plantains had been killed with 2,4-D there was little or no recovery from old plants up to late spring of the following year, since the storage roots were generally completely killed as illustrated in Figure 4 C. Young seedlings generally did not appear in treated plots until after April. In contrast to the response of plantains, there was considerable recovery of



FIGURE 4. Examples of effective top kill of dandelion and broad-leaved plantain treated with 0.1 per cent 2,4-D. (A) Dandelion: left, non-treated control; right, appearance on August 8 of plot treated July 12. (B) Plantain: left, non-treated control; right, appearance on August 8 of plot treated July 12. (C) Broad-leaved plantain (median longitudinal section). Left, normal appearance of fleshy storage root of control; right, appearance of decayed storage root caused by 0.1 per cent 2,4-D spray applied 28 days previously.

dandelion in treated plots two or more months after treatment. Many of the rosettes which appeared were no doubt seedlings less than one year old but some of the plants were found to be rejuvenated rosettes from old roots which had not been entirely killed. Examples of this type of rejuvenation or regrowth are shown in Figures 3 B and 5 B. Close examination was frequently required to distinguish between the newly formed rosettes arising from new seedlings and those rejuvenated from old roots due to the fact that in either case the first leaves produced had the entire leaf juvenile form. Although a multiple rosette is a substantial indication that the plant is two or more years old (6) only one rosette may arise from roots damaged by 2,4-D as illustrated by the plant on the right in Figure 3 B. Final proof as to whether the rosette had grown from the roots of plants more than one year old was obtained in some cases only after digging around the base of the plant or by removing the entire plant.

The size of a dandelion rosette depends upon nutrients and other growth factors and not primarily upon age (6), so that in some instances a plant only three months old from seed, as in Figure 5 C, may appear older than it actually is. Flowering of the greenhouse-grown seedlings had not occurred at the end of four months even though the leaves had the dissected form characteristic of mature leaves. Sears (6) has pointed out that flowering and subsequent branching of crowns constitute reliable criteria for estimating the age of dandelions. The following statements are considered particularly pertinent in this connection (6, p. 429): "At the end of the period of most active flowering (autumn of second year or spring of third) the vigorous production of flower buds forces apart the inner leaves of the original rosette with their axillary growing points. From such growing points arise numerous secondary rosettes. As a rule several of these persist, functioning essentially as new individuals, in spite of their common root connection."

Unless the same plants in treated plots are examined frequently after the first leaves have died, there is a possibility of assigning regrowth to plants which appeared to have died (tops killed) before rejuvenation occurred, when actually the rejuvenation may have occurred before the last leaf died. It frequently happened that one or two badly damaged mature leaves remained attached to the root for several to many days after the majority of the leaves had disintegrated. Such leaves were generally narrow and so badly distorted that they were scarcely recognizable, particularly when low lying and intermingled with the grass.

In general a more rapid killing of dandelion tops resulted from treatment with high concentrations of 2,4-D than with low concentrations in the range 0.04 to 1.0 per cent. There was a similar relationship between concentration of 2,4-D and the relative amount of recovery in the form of regrowth from old roots not entirely killed or of the growth of new seedlings (Tables I and V). One important exception is the recovery in the

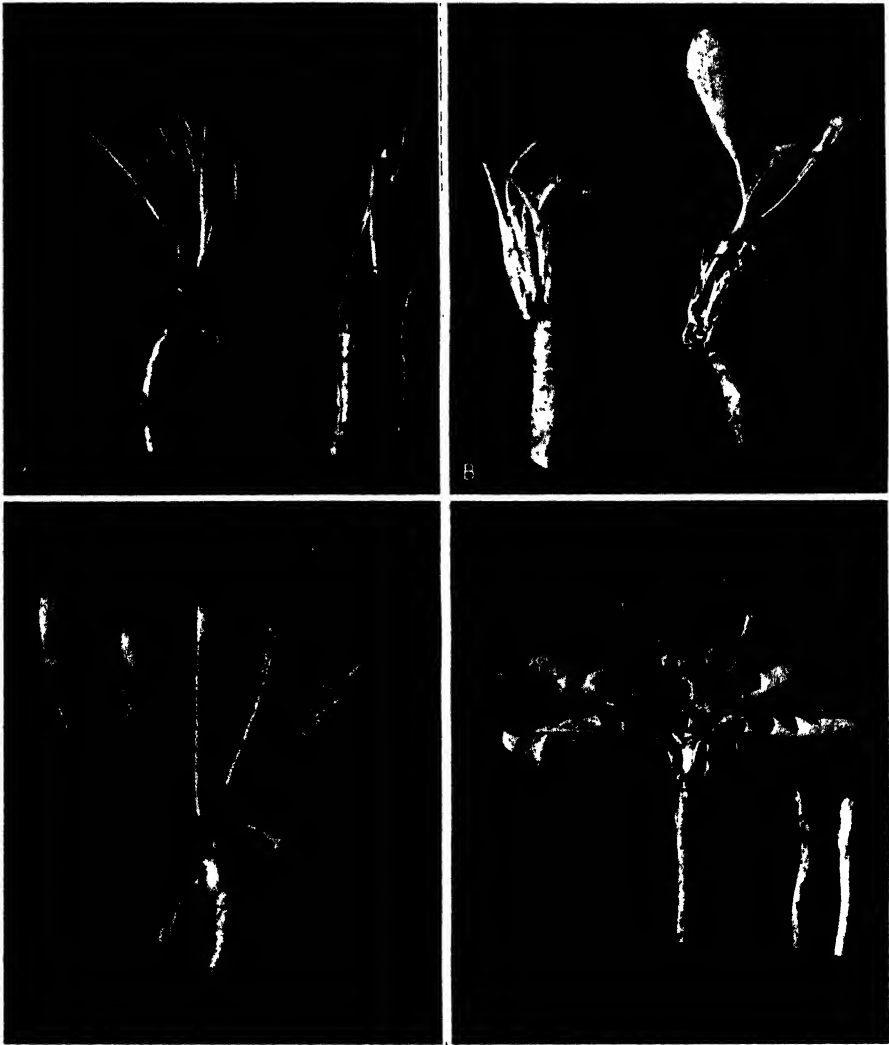


FIGURE 5. Rejuvenation and growth habits of dandelion. Plants removed from control and 2,4-D treated plots June 13, 1947. (A) Left, non-treated plant more than one year old showing normal habit of multiple rosette rejuvenation; right, similar plant badly damaged but not killed by 2,4-D spray applied April 15, 1947, and also the remains of a plant completely killed. (B) Recovery growth from one inch below ground level in the form of etiolated stem-like vertical rhizomes in plot treated June and July, 1946. Note juvenile (entire) form of leaves. (C) Three-month-old seedling grown from seed in greenhouse. (D) Left, control; right, one root soft at top and one root entirely firm after rosettes were killed with 0.2 per cent 2,4-D applied May 12, 1947.

plot receiving 1.0 per cent 2,4-D. In this case 12 dandelions with well developed rosettes were present on May 23, 1947, notwithstanding that the tops of all 22 dandelions and most other plants were killed the previous July 24, 1946 (Fig. 2 A).

The higher rate of application of 0.1 per cent 2,4-D (1 gal. per 90 sq. ft.) was more effective than 1 gal. per 180 sq. ft. for maximum killing in some cases (Table III) but not in others (Tables II and IV). With respect to recovery, the higher rate was more effective in one experiment (Table III) but not in three others (Tables I, II, and IV).

Respraying one to three weeks after the first treatment generally caused a more rapid killing of dandelion tops than the initial treatment (Tables III, IV, V, and VI) but the resprays were not similarly more effective in reducing recovery the following spring. On the other hand, spraying in July and again in September, resulted in little or no recovery the following April (Table IV).

The results recorded in Tables I to VI show that the tops of from 90 to 100 per cent of the dandelions could be killed by 2,4-D under a wide range of conditions, but that recovery of some plants two months or more after treatment was the rule rather than the exception. For example, of the 57 treatments, only 9 were sufficiently effective to prevent dandelion rosettes from appearing in the treated plots in April of the following spring. Records of 28 treatments were kept of the recovery of dandelions in 1946 and in April of 1947. Recovery occurred in 23 plots in 1946 and persisted to April in 22 plots. Differences either in the killing of the tops of dandelions or in recovery in treated plots were no more pronounced for concentration, rate of application, and respraying than for differences due to the date of treatment. Much of the recovery in these particular experiments was no doubt in the form of seedling dandelions, but there was a higher percentage recovery by rejuvenation from roots of established plants than was anticipated on the basis of reports that dandelions are "susceptible" (1, 2, 5). Perhaps the most comprehensive summary of the results with 2,4-D obtained by workers all over the country is contained in the report of The Policy Committee on Herbicides for 1947 (5) which qualifies the "susceptibility" of dandelion by the statement that roots are frequently killed by one application of 2,4-D.

Arriving at any exact percentage of rejuvenation of dandelion rosettes in treated plots was not possible in the present experiments due to lack of labeling individual plants, except in Experiment 6 where dandelions had been transplanted into rows (Table VI). In recent tests (May 12, 1947), labeling of individual dandelion plants has been carried out. It is of interest that rejuvenation started in some cases one month after a 2,4-D treatment which resulted in killing the above-ground parts in less than 30 days. Some of the leaves on these rejuvenated rosettes were modified. Thirty days after treatment there was relatively little rejuvenation although some of the roots were at this time intact as illustrated for two specimens re-

moved from these plots (Fig. 5 D). Within two and one-half months after treatment, the top kill of dandelions was as follows: 10 of 14 were killed by 0.1 per cent 2,4-D, 14 of 15 by 0.2 per cent 2,4-D, 7 of 14 by 0.2 per cent  $\alpha$ -naphthaleneacetic acid, and 6 of 13 by a mixture of equal parts of 2,4-D and  $\alpha$ -naphthaleneacetic acid having a total concentration of 0.2 per cent. The corresponding recovery (rejuvenation) of plants showing top kill was as follows: 4 of 10, 9 of 14, 0 of 7, and 1 of 6 respectively for the four treatments just mentioned. In this case the highest percentage recovery occurred in the plot receiving 0.2 per cent 2,4-D where the percentage top kill was greatest and most rapid. The slowest rate of killing resulted from treatment with  $\alpha$ -naphthaleneacetic acid. There are probably other perennials which respond to 2,4-D much the same as dandelion. The large-leaved Patience dock appears to be one, since in most tests all roots were not killed entirely, with the result that regrowth occurred the same year or the following spring.

There is a general agreement in the literature (1, 2, 5) that most weeds, including dandelion, are most effectively killed when in an active state of growth and that under soil and atmospheric conditions conducive to active growth of weeds, eradication is more rapid and more complete. Our results with dandelion and plantain as well as with certain other species do not appear to conform with this generalization. Tops of rapidly developing dandelions in the spring were never killed as quickly or so effectively as in midsummer in these tests. Hawkweed, cinquefoil, and Japanese honeysuckle were killed more readily under dry poor soil conditions than under conditions which produced vigorously growing plants. Young seedlings of Patience dock were relatively resistant, whereas the tops of older plants were killed to the ground.

On the basis of the results with dandelion, it would appear that spraying in June or July and again in September with 0.1 per cent 2,4-D (1 gal. per 180 sq. ft.) would give the maximum killing with the minimum recovery. In contrast, narrow-leaved plantain requires only one application of 0.1 per cent 2,4-D and broad-leaved plantains will be killed to nearly the extent of 100 per cent but perhaps not completely eradicated by a single application. 2,4-D treatments should be repeated each year if lawns are to be kept relatively free of dandelion and plantains.

Successful use of 2,4-D for eradicating dandelion, and also plantain, should be correlated with the practice of top dressing and seeding if there are numerous holes or bare areas left by the disappearance of these weeds as described in Experiment 1. In regularly cared for lawns where there is a fairly thick stand of desirable grasses, dandelions seldom occur as large plants and hence the holes left after the plant dies are not likely to be large. Regardless of the time of year when dandelions and plantains are most effectively killed, the 2,4-D should be applied when it is most feasible for seeding lawns which is generally in the spring and fall.

The ineffectiveness of 2,4-D for killing dandelions in a poorly drained

lawn (Experiment 7) indicates that soil conditions may be an important limiting factor for the effectiveness of 2,4-D in the killing of other species.

#### SUMMARY

A single treatment with 0.1 per cent 2,4-D was effective in eradicating narrow leaved plantain and two species of broad-leaved plantains with little or no recovery up to April of the following spring. *Plantago rugelii*, the predominating species in the region of Yonkers, N. Y., was more resistant than *P. major*.

The above-ground parts of dandelion were readily killed with a single application of 2,4-D, as in the case of plantains, but recovery responses occurred in most dandelion plots the same year and persisted through the spring of the following year. Recovery responses included new seedlings and rejuvenated rosettes arising from the roots of older plants not entirely killed by the spray treatment.

Although different 2,4-D treatments varied with respect to the rate of killing above-ground parts of dandelions, recovery responses were not influenced by the following factors: concentration in the range 0.05 to 1.0 per cent, rate of applying in the range 1 gal. per 90 to 180 sq. ft., respraying one to three weeks after the initial treatment as compared with a single application, and time of applying the spray.

Treatments applied in July were as effective or more effective for killing the tops of dandelions and also for minimizing recovery, as the sprays applied in May, June, or September. These results are not in agreement with the prevalent conception that dandelions are eradicated more effectively when treated in the spring and fall than when treated in midsummer.

Considering the nature of recovery responses in treated dandelion plots the same year and the following spring, at least one application of 2,4-D a year should be made on lawns in the spring or fall, preferably at both times, when top dressing and seeding are most feasible.

The ineffectiveness of 2,4-D for killing the tops of dandelion in a wet, poorly drained lawn, indicates that soil conditions may be an important limiting factor for the effectiveness of 2,4-D in killing other species.

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